User manual

TReCCA Analyser Version 4.0

Time-Resolved Cell Culture Assay Analyser

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1 Preface

We thank you for choosing the TReCCA Analyser for your time-resolved data. The aim of this manual is to provide you with an overview of the functionalities of the program and a course of action in the case of errors. This manual does not claim to be complete and we welcome any ideas for its improvement.

An article about the TReCCA Analyser has been published in the open access and peer-reviewed journal PLOS ONE (Lochead et al., 10(6):e0131233, 2015). Please refer to this publication for a more detailed explanation of the justification of some of the proposed analysis steps and for a quick illustration of the possibilities of the program.

For more specific information on the actual code of the program or for its further implementation please refer to the code of the TReCCA Analyser which is freely accessible on the website of our research institute in Heidelberg:

http://www.uni-heidelberg.de/fakultaeten/biowissenschaften/ipmb/biologie/woelfl/Research.html.

If you wish to discover the use of the program through a practical example, you may also refer to our tutorials (also available on our website). They will guide you through different analysing exercises that will exemplify the use of the program.

We wish you an enjoyable reading!

2 Intended use

The TReCCA Analyser is conceived to facilitate, speed up and intensify the analysis and representation of your time-resolved data, more specifically in the case of cell culture assays. Without having to type any formula, it will perform at wish the following calculations:

- Control condition normalisation.
- Technical replicate averaging and standard deviation calculation.
- Smoothing and slope calculation of the data in order to obtain the rate of change.
- IC_{50}/EC_{50} determination of a substance in a time-resolved fashion.

In the particular case of an oxygen measurement over time, where a model of linear diffusion of oxygen into cell culture well plates applies, this program can convert the oxygen values measured to the actual oxygen consumption of the cell culture.

In the even more particular case of using the commercially available 24-well oxygen sensor plate, the OxoDish (PreSens Precision Sensing GmbH, Germany), this program will recalibrate the 24 oxygen sensors at the beginning of the experiment making the read-out more homogeneous.

For the users of the commercially available 96-well pH or oxygen sensor plates, respectively the HydroPlate or OxoPlate (PreSens Precision Sensing GmbH, Germany), this program will convert the relative fluorescence intensity data to respective pH or oxygen values.

The results of all these calculations will be automatically plotted using a simple template and allowing an easy, fast and reproducible visualisation of the data. The graphs produced are highly customisable: titles, axis description, legend content as well as sizes and colours, exportation format... can all be modified as wished.

The TReCCA Analyser is of course not restricted to the analysis of the results of cell culture assays and can be used for any time-resolved data that need to be averaged, normalised, derivated and plotted.

3 Installation guide

The TReCCA Analyser runs on every system which is compatible with the freely accessible statistical analysis software R [1], as long as the packages described hereafter are also available on the computer. Some details of the appearance of the program may vary from one system to the other. In order to use the program, R and the corresponding packages have to be downloaded to the computer, as well as the GTK+ widget toolkit. The program is then unpacked to the computer according to the following instructions.

3.1 Downloading R and the complementary packages

The TReCCA Analyser requires R version 2.12.0 or higher, which can be downloaded from the site R-project.org (www.r-project.org/index.html). Choose a CRAN mirror from the country that you are in and follow the instructions for installation. At the end of this step it should be possible to launch R as seen in Figure 1 for Windows and in Figure 2 for Mac.

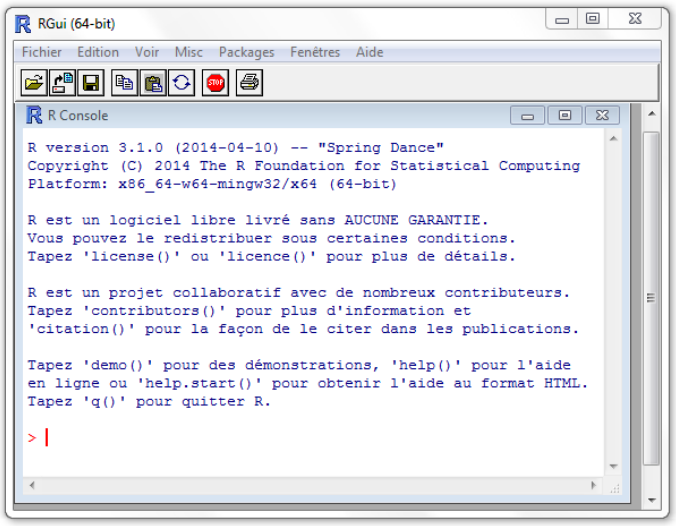


Figure 1: R Console just after launch on Windows

In addition to R, the TReCCA Analyser also requires special packages to run. They can be installed via the package installation guide included in the standard R program.

[1] R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, 2013

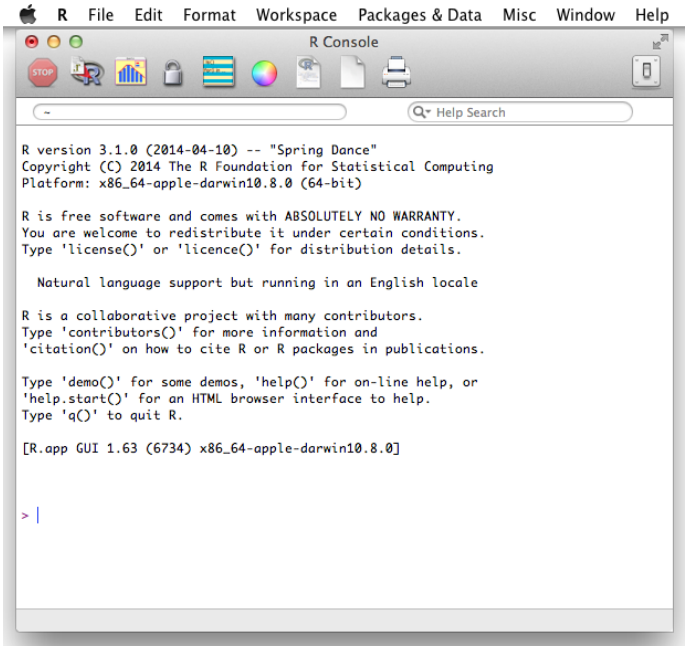


Figure 2: R Console just after launch on Mac

For Windows users, the package installation guide can be reached as seen in Figure 3A, by going to "Packages" and then "Install package(s)". You will be asked to choose a CRAN mirror for download (Figure 3B) and then you can pick which packages to download as seen for the package cairoDevice [2] in Figure 3C.

For Mac users, the package installation guide can be reached as seen in Figure 4, by clicking on "Packages and Data" and then "Package Installer". Pick which packages to download as seen for the package cairoDevice in Figure 4 and click "Install selected".

Installation	Required version	Tested until version	weblink
R software	2.12.0	3.1.1	R Archive
cairoDevice	2.3.0	2.20	Package details
drc	2.3-7	2.3-96	Package details
gWidgets	0.0-46	0.0-53	Package details
gWidgetsRGtk2	0.0-81	0.0-82	Package details
RGtk2	2.12.8	2.20.31	Package details
GTK+	2.8.0	3.6.4	GTK+ combined package
ATK	1.10.0	2.6.0	
Pango	1.10.0	1.30.1	
GLib	2.8.0	2.34.3	
Cairo	1.0	1.10.2	cairographics download

Table 1: Detailed list of all system and software requirements

[2] Michael Lawrence. cairoDevice: Cairo-based cross-platform antialiased graphics device driver., 2011. R package version 2.19

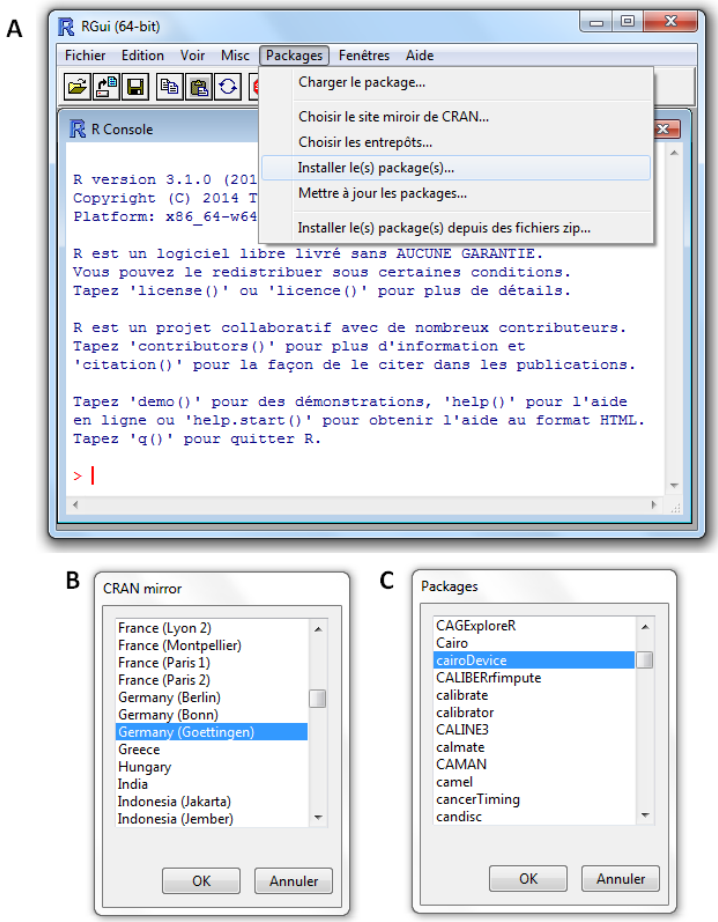


Figure 3: Package installation guide on Windows

You will need to install the packages drc [3], gWidgets [4], gWidgetsRGtk2 [5] and RGtk2 [6] in the same manner as listed in Table 1.

As soon as RGtk2 is installed, it can be loaded by typing `library("RGtk2")` in the R console. You will automatically be asked to install GTK+, if it is not installed already, as seen in Figure 5.

The automatic download will install the whole GTK+ framework together with all the required packages from the all-in-one bundle listed in Table 1 that can also be found on the GTK website (www.gtk.org). Restart R after installing GTK+.

[3] C. Ritz and J. C. Streibig. Bioassay analysis using r. Journal of Statistical Software, 12, 2005

[4] John Verzani. gWidgets: gWidgets API for building toolkit-independent, interactive GUIs, 2012. Based on the iwidgets code of Simon Urbanek and suggestions by Simon Urbanek and Philippe Grosjean and Michael Lawrence. R package version 0.0-52

[5] Michael Lawrence and John Verzani. gWidgetsRGtk2: Toolkit implementation of gWidgets for RGtk2, 2012. R package version 0.0-81

[6] Michael Lawrence and Duncan Temple Lang. Rgtk2: A graphical user interface toolkit for R. Journal of Statistical Software, 37(8):1-52, 12 2010

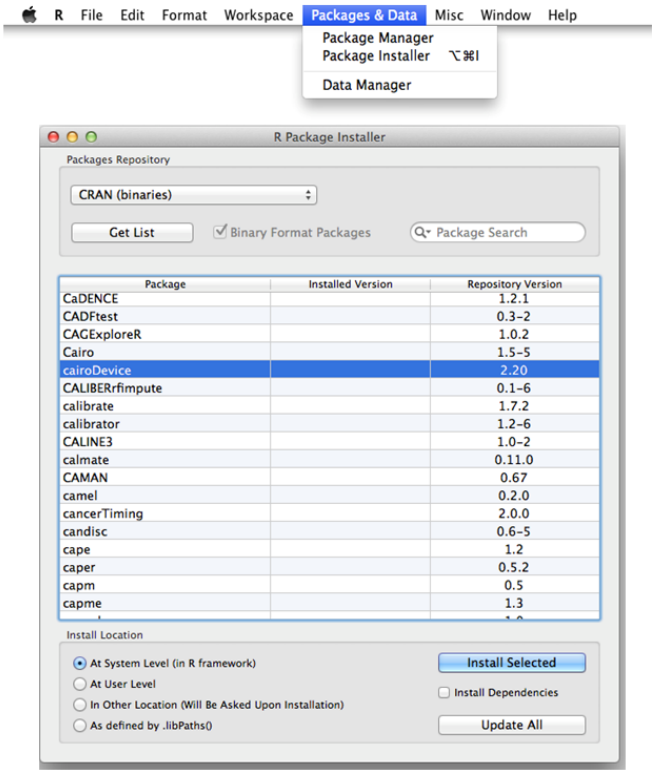


Figure 4: Package installation guide on Mac

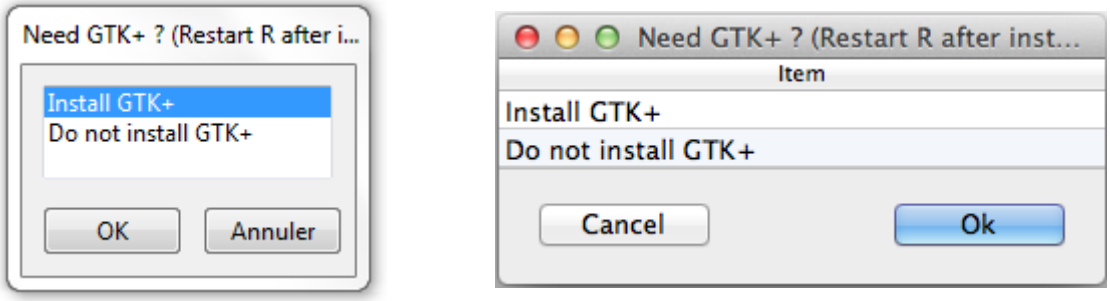


Figure 5: Automatic GTK+ download

3.2 Running the TReCCA Analyser

As soon as R, all the necessary packages and GTK+ are installed, run the program as follows. All the files and graphs produced by the TReCCA Analyser will be saved in a defined *working directory*, which is a folder placed in any convenient place of the computer. The main folder of the program has to be inserted into this working directory and it should also contain the "SampleProject" folder, as well as the "default_settings.txt" and the "qualitycontrol.txt" files, as shown in Figure 6.

In the R console, the path to the actual working directory is given by the command `getwd()`. To set the working directory, type `setwd("path")` and define the path leading to the working directory as exemplified in Figure 7. The working directory folder does

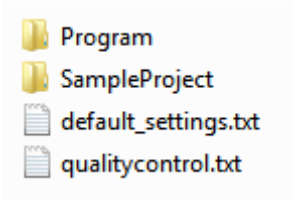


Figure 6: Folder and file content of the working directory

not have to be in the same place on the hard drive every time the program is run, but it should always contain the main folder of the program and the accompanying files.

Once the working directory is set, type `source("Program/mainApplication.R")` to launch the TReCCA analyser, as shown in Figure 7. In the example of this figure, typing `getwd()` gives back the localisation of the actual working directory, in this case the "Documents" file. By typing `setwd("C:/Users/Julia/Desktop/TReCCA Analyser")` the working directory is set to a folder called "TReCCA Analyser" placed on the desktop of the computer. The change of working directory is confirmed by the command `getwd()`. By typing `source("Program/mainApplication.R")` the program is then launched.

```
> getwd()
[1] "C:/Users/Julia/Documents"
> setwd("C:/Users/Julia/Desktop/TReCCA Analyser")
> getwd()
[1] "C:/Users/Julia/Desktop/TReCCA Analyser"
> source("Program/mainApplication.R")
```

Figure 7: Commands to set the working directory and launch the TReCCA Analyser

To make the launching of the TReCCA Analyser easier and faster it is possible to save the two commands needed to change the directory and run the program to a text file, and then copy-paste them to the console when it is started, as shown in Figure 8.

```
setwd("C:/Users/Julia/Desktop/TReCCA Analyser")
source("Program/mainApplication.R")
```

Figure 8: Text file for launching the TReCCA Analyser from the R console

After starting the program, a GTK application should start on your computer and by clicking on the GTK icon on your tool bar, the Welcome screen of the program should appear as shown in Figure 9, indicating where the current working directory is. If the directory is not changed beforehand the program will not be loaded. Always restart the program if the working directory is changed.

If the TReCCA Analyser is not launched, check that your pathways do not contain

any special characters (for example ü, é, Japanese, Arabic characters...).

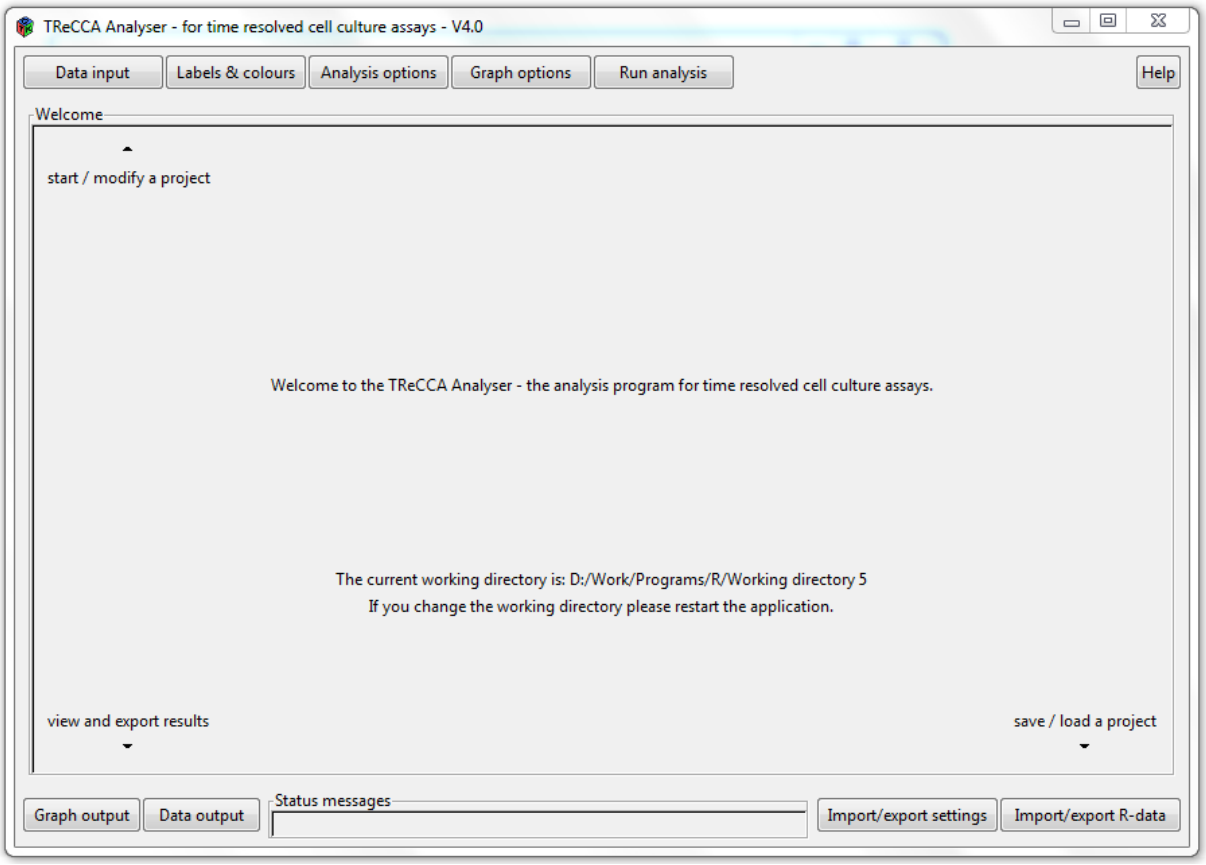


Figure 9: TreCCA Analyser welcome screen

In the console, there will be a message indicating that all R objects were deleted. This is to make sure that there are no conflicts when running the program several times within the same R session. This also means that any progress made in R before starting the program will be lost.

4 Quick guide

This quick guide will give the key steps to follow to use the TReCCA Analyser. Please refer to the more detailed descriptions following in chapter **5.Detailed user guide** for any extra information.

Throughout the use of the program, whenever something is entered in an entry box, it is necessary to press return to process the setting directly, otherwise it will only be processed as soon as it is needed by the program. If you already have a personalised settings file, load it by clicking on Import/Export settings, import the input files and the template and go directly to step 5.

1. Click "Data input"

1. Fill in the Data layout (top left of the screen).
2. Define the Cutting borders (bottom left of the screen).
3. In File import, select the right separators and file path(s). The file should contain the time points in the first column and the rest of the data in the following columns, each headed by a unique column name.
4. Click the "Import Files" button and solve status messages if necessary (bottom middle of the screen).

2. Click "Labels & colours"

1. Load a previous template with "Load template" or follow the next points.
2. Auto-fill the template by clicking "Autofill labels", "Autofill numbers", "All black" and "All solid".
3. Export the template by clicking on "Save template" and edit it with any spreadsheet application (without changing the first row).
4. Import the modified template with "Load template".

3. Click "Analysis options"

1. Select the analysis you want to run on your data using the tick boxes.
2. Fill out the settings for each chosen analysis (bottom half of the screen).

4. Click "Graph options"

1. Choose all the titles for the graphs.
2. Enter the axis labels and limits. All the graph options can be changed after the analysis is run, once the graphs are made.

5. Click "Run analysis"

1. Chose the name of the results folder in which the results will be saved.
2. Wait for the analysis to be run. The analysis time should be under 15 minutes.

6. Customise the graphs

1. On the right you can switch through the different graphs.
2. If you wish to visually exclude some lines, click on their tick boxes to the left of the screen and then on "Refresh lines".
3. If you wish to exclude some conditions from the analysis, go back to the template and name them "Exclude". You will have to rerun the analysis by clicking "Run analysis" for the changes to be taken into account.
4. Customise the graphs by using the options displayed at their bottom (point size, error and grid intensity, the legend position and format...).
5. You can also change the settings in "Graph options" menu and apply them by clicking "Refresh options".

7. Export graphs and data

1. Export each graph by clicking on the "Export displayed diagram" button, and enter a file name and size in inches. It will be saved in the result folder.
2. To save the data as .csv files click on "Data output" and select which data sets to export, their name and click on "Export Files".
3. By clicking on Import/export" R-data, you can save the R-data so that you will not have to rerun the analysis to change the graph customisation.
4. By clicking on "Import/export" settings you can save the settings for the next analysis.

5 Detailed user guide

In this part of the manual, the TReCCA Analyser is described in full detail, with an overview of all the modifiable options and the consequences of their selection. The buttons at the top of the screen displayed in Figure 10 should be filled in one after the other and will be successively described in this part of the handbook.

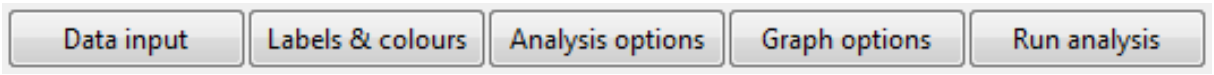


Figure 10: Buttons of the main tabs of the TReCCA Analyser

5.1 Data input

When starting the program, the first step consists of importing the data. Click on the "Data input" button in the upper menu bar to see the tab displayed in Figure 11. It consists of three subunits: "Data layout", "Cutting borders" and "File import", described more precisely hereafter.

To have a second look at data previously imported, analysed and saved as R-data, click "Import/export R-data" and load the corresponding file.

If the settings from a previous analysis or from a similar experiment were saved, it is also possible to import them by clicking "Import/export settings" and loading the corresponding file. Clicking through the different settings is still possible to check that they are set as wished and they can be modified if necessary. Even after importing the saved settings, it will be necessary to load the template again. When importing R-data it is also essential to first import the settings so that the interface is correctly set for the imported data to be shown.

5.1.1 Required format

The format required for the input files is .csv or .txt. It is possible to convert files to these formats with usual spreadsheet applications (for example excel files .xls or .xlsx) using the "save as" function. In each document, the first column has to be the time column (after cutting off the edges). Following this column there can be as many columns as desired, each containing the measured data from one condition (typically, from one well). The first line after the file header must contain unique names for each column. An example of

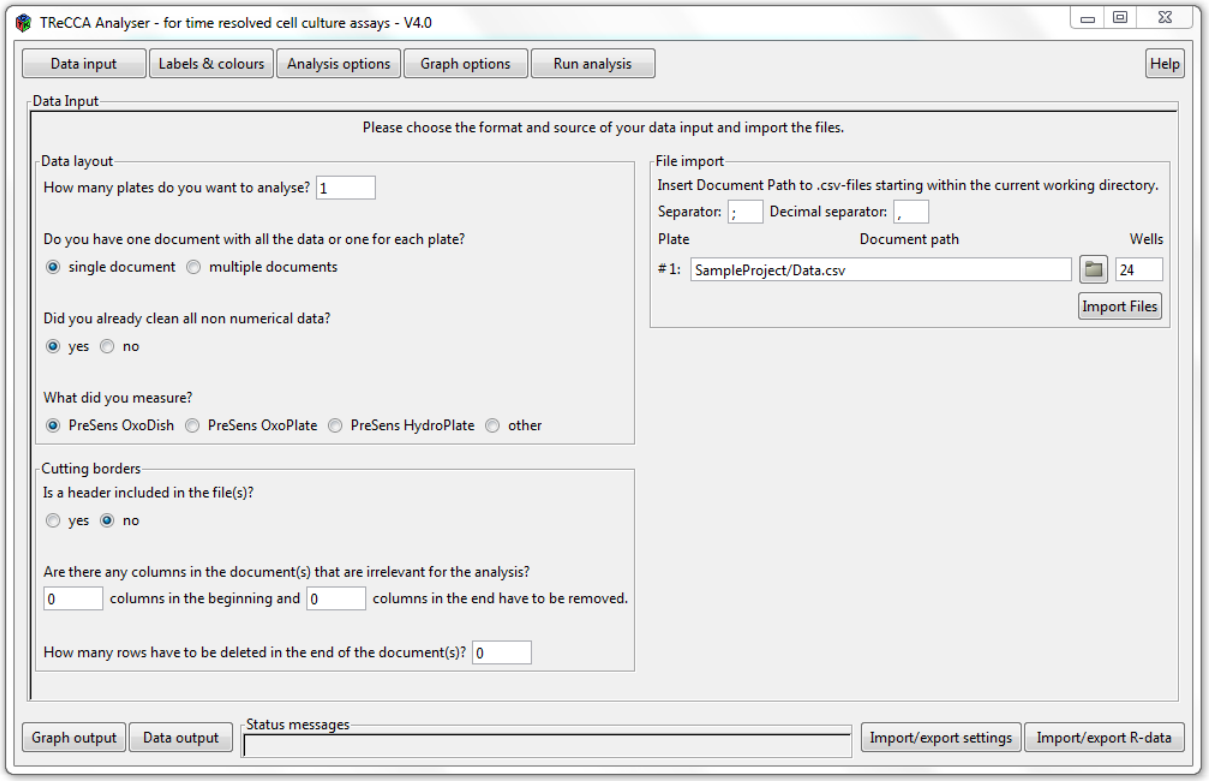
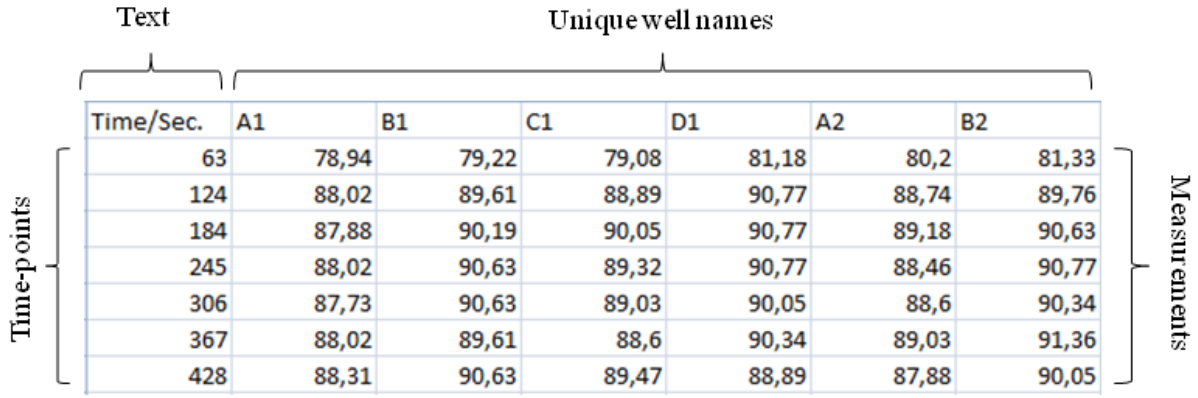


Figure 11: Data input tab

input file format can be seen in Figure 12.



Time/Sec.	A1	B1	C1	D1	A2	B2
63	78,94	79,22	79,08	81,18	80,2	81,33
124	88,02	89,61	88,89	90,77	88,74	89,76
184	87,88	90,19	90,05	90,77	89,18	90,63
245	88,02	90,63	89,32	90,77	88,46	90,77
306	87,73	90,63	89,03	90,05	88,6	90,34
367	88,02	89,61	88,6	90,34	89,03	91,36
428	88,31	90,63	89,47	88,89	87,88	90,05

Figure 12: Required input file format

5.1.2 Data layout

The first parameter defining the data layout is the number of plates, which also regulates the number of input lines in the "File import" (to the right of the screen). The maximum number of plates which can be analysed at once is 10.

If there are several plates to be analysed, it is possible to either have all the plates

in one single document with one time column for all, or have the plates in multiple documents with individual time columns. In this last case, it is important that each file contains exactly the same time points. Select "multiple documents" and this option will change the number of paths that can be given in the "File import".

Since non numerical values in the files (such as "error", "NA" or "NAN") will disturb the analysis they all have to be removed. This can either be done manually in a spreadsheet application, or the program can replace all the non numerical values automatically with 0. For this, select "no" for the previous accomplishment of non numerical data cleaning and when importing the data the first pop-up window in Figure 13 will appear. It might be the case that certain character strings force the program to replace whole columns with 0. When the data import is finished there will be a message indicating whether there were no replacements, discrete replacements or column replacements and how many, as seen in the rest of Figure 13. If there are whole columns replaced the first five rows of the data will be printed into the R console so that it is easy to identify the columns containing character strings.

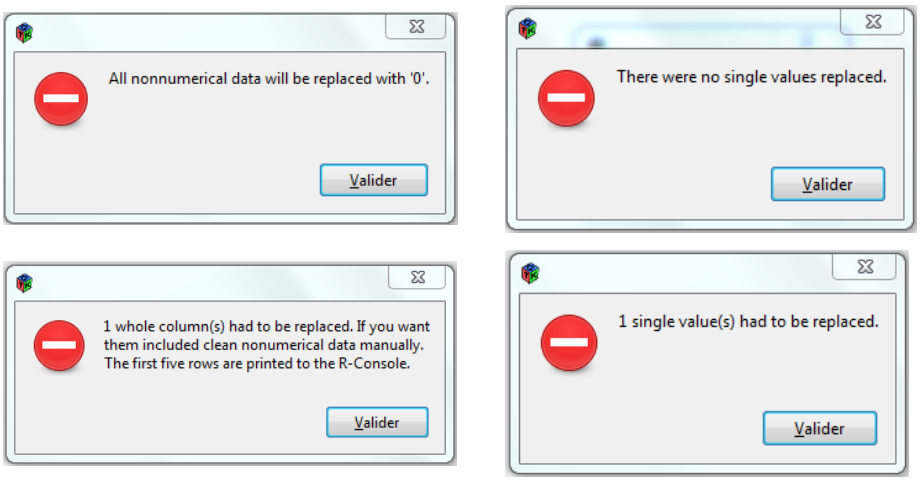


Figure 13: Pop-up windows after clicking "Import Files"

Finally, select which kind of measurement has been done, in order to have access to the specific analysis options further on in the program. The default option is "other", select one of the other options in the case of a PreSens OxoDish, PreSens OxoPlate or PreSens HydroPlate measurement.

5.1.3 Cutting borders

Since most of the documents written by the measurement software will not only contain the actual data, but also additional information (date of the measurement, wavelength chosen, identification number...), it is possible to cut off the extra file lines and columns automatically using the program. This way it will not be necessary to delete the extra

data using classical spreadsheet applications.

If there is a header in the file(s) (not taking into account the first line of the file which belongs to the data, see Figure 12), select "yes" and whether it should be saved to a separate file or just be removed. Either way the original file will not be changed, just the imported version. If you chose to save the header, a .csv file called "Header_exported.csv" will be saved after the analysis is run in the result folder that you will name. The number of rows contained in the header should be equal to the number of rows in the spreadsheet program used. In the exceptional case of Excel files containing Excel-reports, it will be necessary to count the rows in a text file, as their number will increase with the report. Empty lines between the header and the data also count as headers and have to be taken into account.

In a similar way, if there are columns in the beginning or in the end of the files (error messages, time stamps, time columns in other units...) they can also be removed automatically by the program. To do this, enter the right numbers in the respective fields, without forgetting to take the empty columns into account.

The same thing can be done also with rows at the end of the document, once again not forgetting to take the empty rows into account.

5.1.4 File import

First fill in the cell separator and decimal separator. The cells have to be separated by some character string that is not a white space and the decimal separator for numbers can be any single character.

To choose the files, either browse the system by clicking on the button next to the input line, or type the path to a file lying within the current working directory.

Fill in the number of wells per plate, which will determine how many columns are expected from the imported data. The number of wells per plate is important in order to perform the sensor correction or to normalise the plates independently to one condition present in each plate. To normalise several plates to a condition only measured in one of the plates, copy-paste them together into one big file and enter the sum of the wells as the well number to be analysed.

5.2 Labels & colours

Click on the "Data input" button in the upper menu bar to see the tab displayed in Figure 14. In this tab, the labels and colours for each column of data (or well of the plate) are determined and defined into a template.

This information will be used during the data analysis to determine which conditions should be averaged, which ones should be used for normalisation, which ones for IC_{50} determination, or in the case of the OxoDish, for sensor correction.

The information from the template is also used for plotting, as the colour and line type of each well are determined, and to set the legend of the graphs. The template can be modified directly in the interface of the TReCCA Analyser, but for making major changes in a template, it is probably faster to export the template to a spreadsheet application and modify it there.

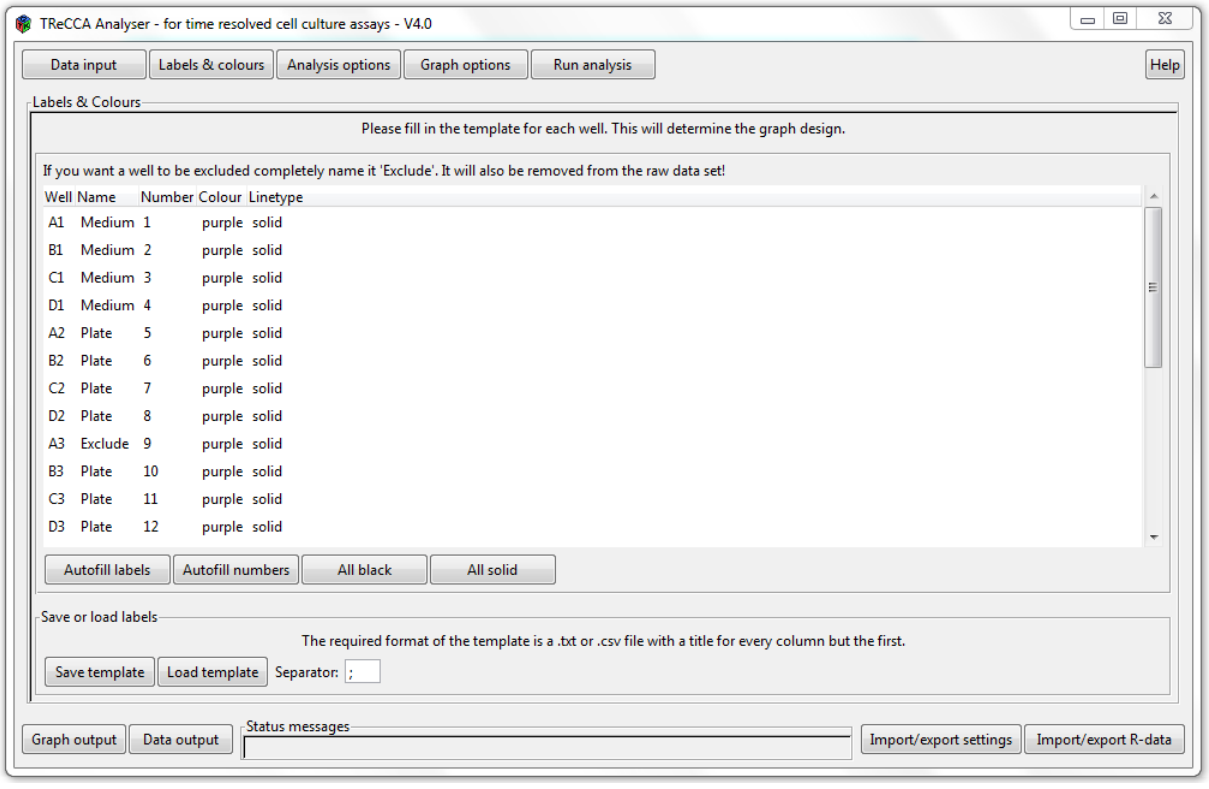


Figure 14: Interface of the Labels & colours tab (sample template loaded)

5.2.1 Template layout

The template is a table containing 5 columns, which are "Well number", "Name", "Number", "Colour" and "Line type". The Well numbers must be the same as the names of the columns of the data sets in the imported data (from example A1 to D6 or 1 to 20).

These can be detected automatically and added to the template by clicking the "Autofill labels" button.

The Name can be any type of character string, but many special characters will not be recognised or will cause problems depending on the system running. This name will be used for the legend labels and to determine groups of wells for averaging for example.

Instead of using the "Name", it is also possible to use the "Number" column to define groups of columns/wells. The oxygen calibration pop-up also determines the groups of wells using this number. If all the numbers in the template are the same, the legends of the graphs will be sorted alphabetically. By assigning each condition a number, it is possible to determine in which order each label will appear in the legend; the name assigned the smallest number will be displayed first and so on. As a general rule, in the case of the legend for average conditions, the "Number", "Colour" and "Line type" of the first occurring sample of a specific group will be used to represent the average condition. For example, if Well 1 has the name "Medium" and the colour "red", and Well 2 has the name "Medium" and the colour "blue", then when representing the "Medium" average the line will be red.

The "Colour" must be a character string referring to one of the 657 R colours. A list of the available colours can be found online (by searching "R colours") or by typing `colours()` in the R console to get the available list. The "Line type" can be "solid", "dashed", "dotted", "dotdash", "longdash", "twodash" or "blank" (not be visible).

5.2.2 Filling the template automatically

When creating a new template, using the auto fill options will make sure the template has the right format. The "Autofill labels" button will detect all the column names in the input data and place them in the Well column. This requires the data to be loaded already.

Make sure that all the column names are different inside one imported file. When importing data from two separate files, all the Well names of the second file will be modified to display ".1" at their end. In this way, it is possible to import files with identical column names without having to change these names manually. In the case of importing three files which all have A1 as first column name, these will appear as A1, A1.1 and A1.2 in the "Well" column of the template. The "Autofill labels" button will automatically fill the Colour column with "black" and the rest of the template with "0", so this button should be the first used and be sure to save the template displayed beforehand if necessary.

The "Autofill numbers" button will give a unique number to all the wells. "All black" and "All solid" will set all the colours to black and all the line types to solid respectively.

5.2.3 Saving and loading

As mentioned before, even if it is possible to modify the template directly in the TReCCA Analyser interface, it is probably easier to use a classical spreadsheet program for major changes, as for example after using the auto fill options. To save the template click the "Save template" button and type a file name ending with .txt or .csv. It is also possible to type a path to a different folder within the current working directory (for example, "Templates/NewTemplate.csv"). The template can then be changed at will and loaded again once it is completely filled. To load a previously saved template, click on the "Load template" button, which will open the file chooser. When importing a template, be sure to specify the right cell separator. After importing saved settings, the template will have to be loaded again from the file system for the program to run smoothly.

5.2.4 Excluding data from the analysis

In order to exclude one well from the data analysis (if you realise that it is an outlier condition for example), it is possible to type "Exclude" as its name, thus bypassing the more time-consuming step of actually deleting the column from the original data set and then from the template. Naming a condition(s) "Exclude" will automatically remove the column(s) from the data set before any analysis is run, so it will not be taken into account for the average calculation, IC_{50} determination, oxygen consumption determination... In the graphs of the raw data though, the conditions may be represented in the wrong colour as the template is not automatically modified. If it is important for the individual conditions to be represented in the right colour, then it will be necessary to delete the condition from the .csv file and the template. Either way, it is possible to access the reduced data set as "Raw data" after the analysis is run.

5.3 Analysis options

In the "Analysis options" tab displayed in Figure 15 the analysis to be performed on the data are selected and the corresponding settings are filled in. First fill in the "Analysis selection" (top part of the screen). As the boxes are ticked, new fields to fill in will appear in the bottom part of the screen corresponding to each possible analysis and the respective settings that have to be filled in. These settings are described in the following paragraphs and for a more precise description of the mathematical calculations that take place, please refer to chapter **6 Technical details**. An overview of the possible analyses and their relations is displayed in Figure 16.

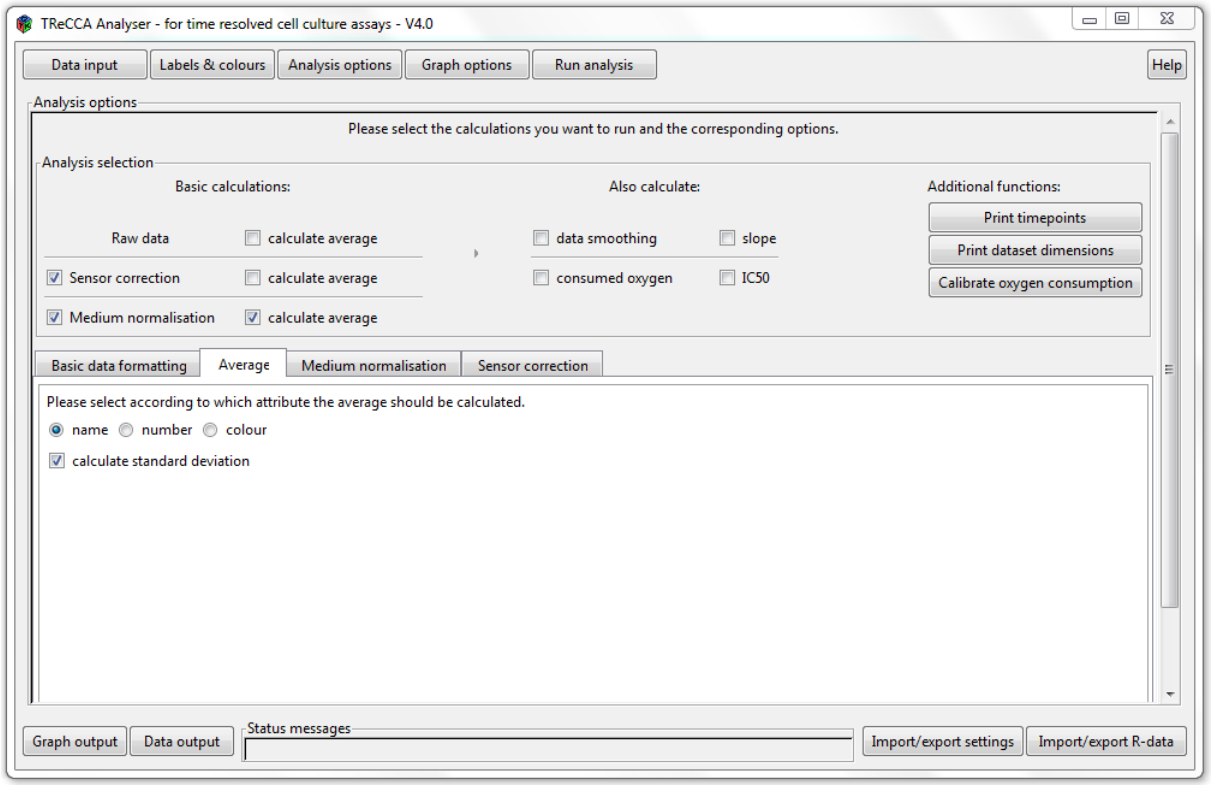


Figure 15: Analysis options tab with the basic data formatting option

Each analysis tab has a "Rerun xxx only" button which starts the analysis of only the actual analysis in order to save time and prevent having to run the whole analyses repeatedly. By pressing a "Rerun xxx only" button, a new window will appear asking if the analysis based on this calculation should also be updated (refer to Figure 16 for an overview of the dependencies). For example, if the normalisation target value is changed and the "Rerun normalisation only" button is pressed, then the TReCCA Analyser will ask whether the average normalisation calculation, which is based on the normalised data should also be accordingly recalculated.

5.3.1 Basic data formatting

The "Basic data formatting tab" is always displayed in the "Analysis options" window, as seen in Figure 15. The first half of the tab has to be filled in accordance with the time-resolved experiment, the second half is optional.

In the first row, tick the time unit of the input data. The time unit used for all the output graphs can be chosen in the second row and the TReCCA Analyser will automatically perform the corresponding unit conversions, if necessary. The output time scale unit will also have an influence on the slope calculation of the data (see part **5.3.8 Numerical**

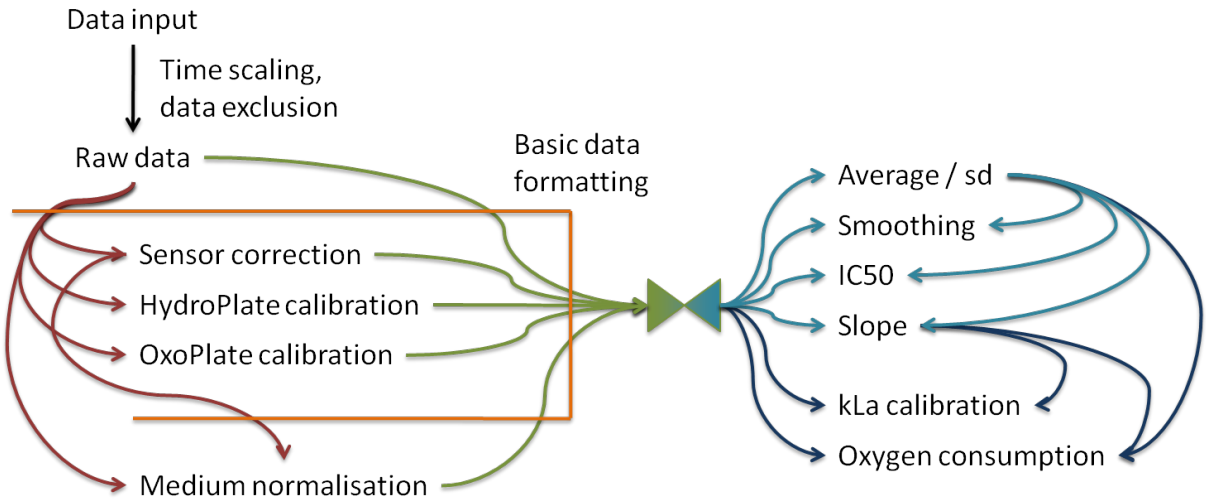


Figure 16: Analyses available in the TReCCA Analyser and their relationship

slope).

The second half of the tab is for performing more advanced data formatting. First the data set used for the reformatting is selected. Any data set that is calculated on the basis of a formatted data set will also be formatted in the same manner (see Figure 16 for a representation of the dependencies between analyses). For example, shifting the time scale of the raw data will imply that the time scale of the normalised or averaged raw data will also be shifted.

To remove the data before or after a certain time point, fill in the corresponding boxes. The time has to be given in the output unit chosen in the first part of the "Basic data formatting" tab. If the times are only available in the input unit, use the "Print time points" button at the top right of the program window. This way, all the time points of the data set will be printed in the R console in two columns with the input and output unit automatically. It is important to note at this point that the data will simply be removed before or after the time points, but the actual value of the time points will stay the same.

To actually transform the time scale, fill in the next lines once again in output units. The given m value will be multiplied to all the points of the time scale (for example, to have the time scale in years and with "day" as output unit, choose m=1/365.2425) and the given b value will be added to all the points of the time scale. The latter is useful if deleting the first part of the data for example. In contrary to the shifting possibility of the second row of the advanced data formatting, here the time points will be taken away and the beginning of the time scale will be set to 0.

The last box will multiply all the data by a given number allowing a conversion of the measurement unit. In this way, it is possible for example to convert data from milimolars to molars by entering 1000.

5.3.2 Average and standard deviation

The "Average option" tab can be seen in Figure 17. Select the criteria from the template in "Labels & colours" to use for the average calculation. The TReCCA Analyser can either average conditions having the same "Name", "Number" or "Colour". The arithmetic mean and the standard deviation will be calculated accordingly for every time point. If only one replicate is available, then the TReCCA Analyser will just take the actual value of that replicate; the standard deviation in this case will be set to 0.

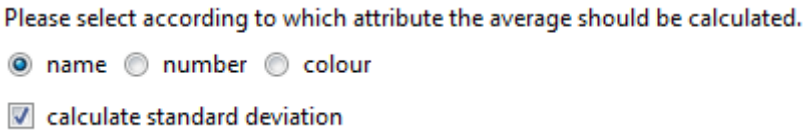


Figure 17: Analysis options - Average tab

5.3.3 Normalisation

The TReCCA Analyser can be used to normalise all the measurements at each time point to a specific condition. For example, in the case of an oxygen measurement over time, a well containing only medium and placed in an incubator should have a stable read-out throughout the measurement. As this can differ slightly over time (slight fluctuations of oxygen in the incubator, drift of the sensors), normalisation can reduce the fluctuations. This analysis step can also be used to normalise all the conditions of an experiment to a non-treated control. The "Normalisation" tab is displayed in Figure 18.

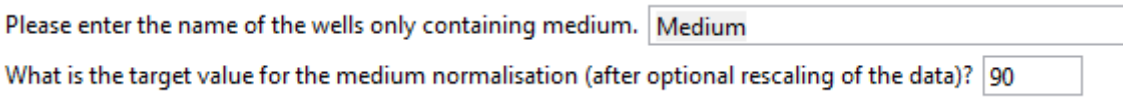


Figure 18: Analysis options - Normalisation tab

Enter the exact name (with capital letters or not) assigned to the normalisation condition in the "Labels & colours" template, and give the target value for the normalisation (this value could be 100 to get percentages in the case of viability studies and a non-treated control normalisation). The normalisation is performed once per plate, which means that each condition in a plate is normalised to the normalisation condition present in the same plate. To normalise to the overall average normalisation condition, merge the

data first in a spreadsheet application and import them as one file.

5.3.4 OxoDish sensor calibration

This option is only available once "PreSens OxoDish" has been selected in the "Data input" window in the "Data layout" part. It is displayed in Figure 19.

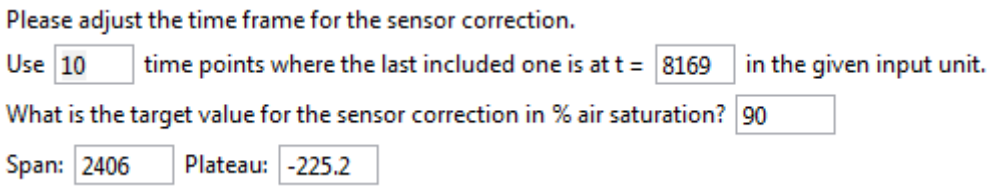


Figure 19: Analysis options - OxoDish sensor calibration tab

When measuring each sensor of an empty 24-well OxoDish (PreSens Precision Sensing GmbH, more information is available in the SensorDishes & SensorVials instruction manual), a read-out difference of between 2 and 8% can be noticed between each sensor, although when being empty the value should be exactly the same. This slight difference can be reduced thanks to the TReCCA Analyser. To do so, first measure the empty sensor plate under the experimental conditions (temperature, humidity...) on the SDR Reader that will be used later for the experiment, until the readout is stable for at least 5 time points. This will give information about the average offset value for each well, which can then be set to a theoretical target value (for example 100% of air saturation if working in the lab or 95% of air saturation if working in an incubator with 5% CO_2). After the sensor calibration, all the wells will start at a much more similar value. For more calculation details, please refer to part **6.3 OxoDish sensor calibration**.

First fill in how many time points should be used for the sensor correction (once the read out is stable, 10 points is a good number) and enter the time point of the last of these time points in the input unit. The TReCCA Analyser will take the value of the time point given and for example the 9 time points before this one, to calculate the basis for moving the whole dataset to a target starting value which must also be given. Span and Plateau are two values which determine the exact form of the calibration curve for each OxoDish lot. Some Span and Plateau values are presented in Table 2. As a rough estimation it is also possible to work with the default settings.

5.3.5 OxoPlate oxygen conversion

This option is only available once "PreSens OxoPlate" has been selected in the "Data input" window in the "Data layout" part. It is displayed in Figure 20.

OxoDish lot number	Span	Plateau
OD-1437-01	2168	-201.9
OD-1407-01	2062	-191.1
OD-1333-01	2081	-194.5
OD-1319-01	2406	-225.2
OD-1309-01	2034	-187.8
OD-1308-01	2026	-188.5
OD-1245-01	2290	-215.0
OD-1228-01	2193	-209.8
OD-1220-01	2226	-208.6
OD-1142-01	2123	-200.5
OD-1133-01	2161	-201.6
OD-1120-01	2105	-201.7
OD-1107-01	2010	-187.6
OD-1045-01	2079	-194.6
OD-1030-01	2208	-210.0

Table 2: Span and Plateau values for different SDR lots

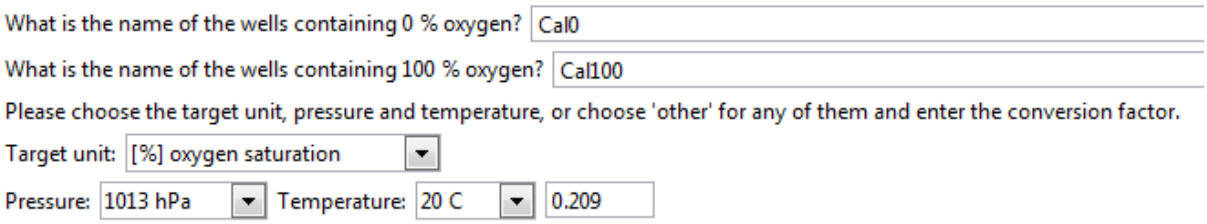


Figure 20: Analysis options - OxoPlate oxygen conversion

When using an OxoPlate (PreSens Precision Sensing GmbH) with a classical spectrophotometer, the emission intensity of a luminophore that is quenched by oxygen is measured in comparison to the emission intensity of a reference luminophore (see the OxoPlate instruction manual on the PreSens homepage for more precise information). The ratio of the indicator luminophore over the reference luminophore then has to be converted by the user to actual oxygen values using two calibration solutions: one containing a 100% of oxygen, Cal100, and one containing a chemical which depletes all the oxygen present by reacting with it, Cal0. This calibration step can be done once for each lot of OxoPlates or once for each plate and during the whole course of the experiment. The time-resolved calibration of each plate separately leads to more precise and less noisy results.

Whatever the calibration method, the TReCCA Analyser will convert the relative emission intensity data automatically, and if available in a time-resolved manner, according to the formulas described in the OxoPlate user manual. For more calculation details,

please refer to part **6.4 OxoPlate oxygen conversion**.

First enter the exact names assigned in the template in "Label & colours" for the 0% and 100% oxygen calibration solutions (often Cal0 and Cal100). If the lot of plates is pre-calibrated, add two columns to the data where each one contains the Cal0 and Cal100 average value for conversion for each time point of the data. If there are several columns with the calibration solutions, the data will be converted according to the average of those conditions. The conversion will be done per imported plate, so for all the data to be converted according to the overall average conditions of all the plates, merge the data in one file.

Select the desired oxygen unit for the output data, according to the ambient temperature and pressure. If the assay conditions differ from the given options, please choose "other" for either of the conditions and enter the unit conversion factor manually in the last slot. To calculate the unit conversion factor, an excel sheet is provided under "Tools and utilities" on the PreSens homepage.

5.3.6 HydroPlate pH conversion

This option is only available once "PreSens HydroPlate" has been selected in the "Data input" window in the "Data layout" part. It is displayed in Figure 21.

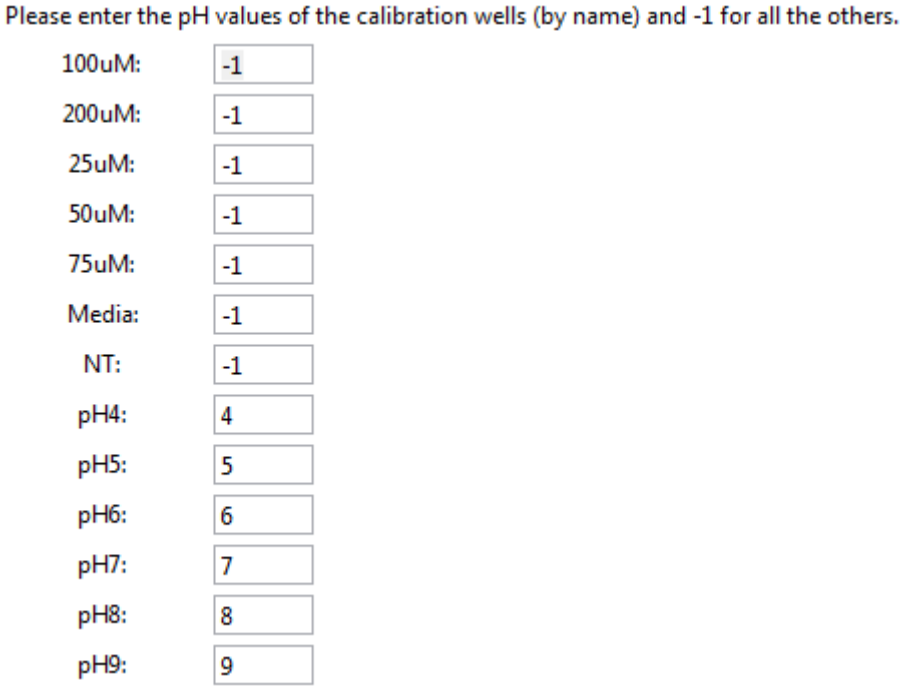


Figure 21: Analysis options - HydroPlate oxygen conversion

When using a HydroPlate (PreSens Precision Sensing GmbH) with a classical spectrophotometer, the emission intensity of a luminophore that is quenched differently depending on the pH is measured in comparison to the emission intensity of a reference luminophore (See the HydroPlate instruction manual on the PreSens homepage for more precise information). The ratio of the indicator luminophore over the reference luminophore then has to be converted by the user to actual pH values using six calibration solutions at pH values between 4.0 and 9.0. The calibration can be done once for each lot of HydroPlates or once for each plate and during the whole course of the experiment. The time-resolved calibration of each plate separately leads to more precise and less noisy results.

Whatever the calibration method, the TReCCA Analyser will convert the relative emission intensity data automatically, and if available in a time-resolved manner, according to the formulas described in the HydroPlate user manual. For more calculation details, please refer to part **6.5 HydroPlate pH conversion**.

For each name of the template that appears in the "HydroPlate conversion" tab, enter the corresponding pH value and -1 for the wells that are irrelevant to the HydroPlate pH conversion as seen in Figure 21. If you pre-calibrated the lot of plates, add columns to your data where each column contains a pH relative intensity average repeated for each time point of the data. If there are several columns with the calibration solutions, the data will be converted according the average of those conditions.The conversion will be done per imported plate, so for all the data to be converted according to the overall average conditions of all the plates, merge the data in one file.

5.3.7 Data smoothing

The TReCCA Analyser can be used to smoothen the data, which is especially necessary to reduce the data noise before calculating the slope of the data (see part **5.3.8 Numerical slope**). For smoothing, each data point will be replaced by the average of the actual data point and of its surrounding neighbourhood. The "Data smoothing" tab can be seen in Figure 22.

First choose the number of points to be selected in each neighbourhood. This should always be an odd number so as to include the actual data point and the same number of time points on either side of the actual data point. It is important to note that the total number of time points in each data set will be reduced after smoothing; if n is the neighbourhood number then $(n-1)/2$ time points will be missing from the beginning and the end of the data set. Also, the bigger the neighbourhood, the smoother and less

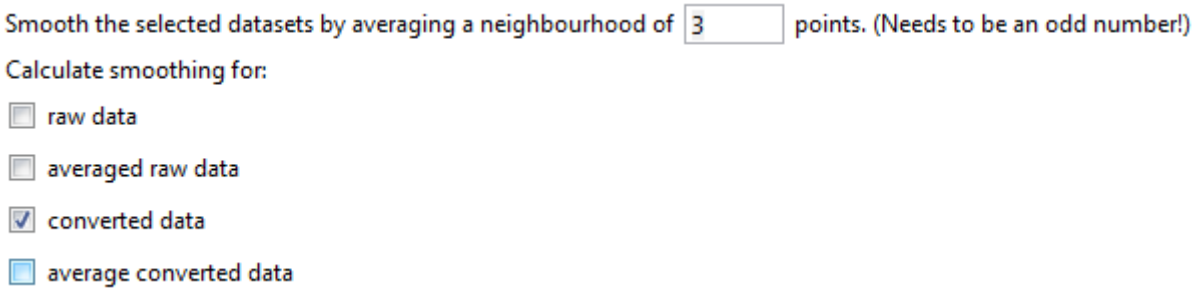


Figure 22: Analysis options - Data smoothing

precise the data will get.

Select the data set(s) that should be smoothed by the TReCCA Analyser by ticking the corresponding boxes. For more details about the formulas used for the smoothing calculations, please refer to part **6.6 Data smoothing**.

5.3.8 Numerical slope

Calculating the slope of time-resolved data can provide valuable information as it will highlight the changes in the speed of the observed phenomenons rather than their actual value. It is important to note that the unit of the calculated slope will be the measurement unit divided by the output time unit. The y-axis label of the slope graphs will have to be changed manually by filling in the "Y-axis label" in the "Graph options" tab (please refer to part **5.4 Graph options**). In many cases, it can be useful to smoothen the data before using it for slope calculation, as noise could hide the actual data trends. The "Slope" tab can be seen in Figure 23.

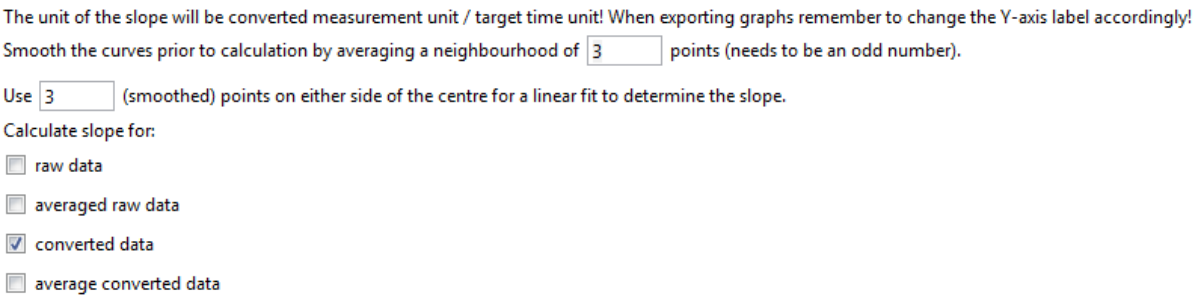


Figure 23: Analysis options - Numerical slope

Fill in the number of points to be included in each neighbourhood as explained in part **5.3.7 Data smoothing**. To use non-smoothed data, fill in the number 1. The slope of each data point will be determined by performing a linear fit of this point and n number of (smoothed) points on either side of it. Select the number of points to be

used for linear fitting, which will determine how precisely the slope should be determined for each data point. It is important to note that the total number of time points in each data set will be reduced after slope calculation; if n is the number of points on either side, then n time points will be missing from the beginning and the end of the data set. Finally, select the data set(s) for which the slope should be calculated by the TReCCA Analyser by ticking the corresponding boxes. For more details about the formulas used for the slope calculations, please refer to part **6.7 Numerical slope**.

5.3.9 Oxygen consumption

The TReCCA Analyser will convert measured oxygen values to actual oxygen consumption values, for all experimental set-ups that fit the model used. The diffusion of oxygen into the liquid should be approximated linear, with the sensor being at the bottom of the well and the lateral diffusion of oxygen being negligible. The calculations implemented here are based on previous publications where the assumptions of the model are described in more precisely [1] [2] [3]. For more details about the formulas used for the oxygen consumption, please refer to part **6.8 Oxygen consumption**.

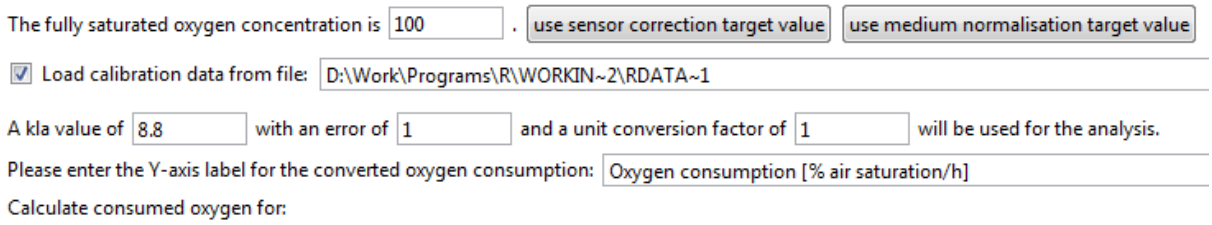


Figure 24: Analysis options - Oxygen consumption

The "Oxygen consumption" tab can be seen in Figure 24. First fill in the oxygen concentration of the fully saturated environment (this would typically be 100% of air saturation for a measurement in the lab or 95% of air saturation for a measurement in the incubator with 5% CO_2). If this is the same value as the one used for sensor correction (for the OxoDish users) or for medium normalisation, click the corresponding "use sensor correction target value" and "use medium normalisation target value" buttons.

[1] K. Eyer, A. Oeggerli, and E. Heinzle. On-line gas analysis in animal cell cultivation: II. methods for oxygen uptake rate estimation and its application to controlled feeding of glutamine. Biotechnology and Bioengineering, 45(1):54-62, 1995.

[2] R. Hermann, M. Lehmann, and J. Büchs. Characterization of gas-liquid mass transfer phenomena in microtiter plates. Biotechnology and Bioengineering, 81(2):178-186, 2003.

[3] G. John, I. Klimant, C. Wittmann, and E. Heinzle. Integrated optical sensing of dissolved oxygen in microtiter plates: a novel tool for microbial cultivation. Biotechnology and Bioengineering, 81(7):829-836, 2003.

The experimental set-up first has to be calibrated in order to determine the diffusion constants. This can either be done using the TReCCA Analyser (See part **5.3.10 Oxygen consumption calibration** of the manual) and loading a calibration file which will automatically fill in the following fields, or by filling in the fields manually for the oxygen mass transfer coefficient k_La value and error. It is essential that the time unit used for the k_La value and for calculating the slope of the data be the same.

To change the unit of the oxygen measurement, fill in the corresponding unit conversion factor. To calculate the unit conversion factor, the excel sheet provided by the company PreSens GmbH under "Tools and utilities" on the PreSens homepage can be useful. Enter the Y-axis label that should be used for the converted oxygen consumption and choose the data that should be used for the oxygen consumption calculation.

5.3.10 Oxygen consumption calibration

In order to convert the oxygen measurement data to actual oxygen consumption data, it is necessary to first calibrate the system and determine its oxygen mass transfer coefficient k_La. The use of this constant is further detailed in part **5.3.9 Oxygen consumption**.

Under the experimental conditions, deplete the oxygen in each well by using either a chemical that will react with oxygen to deplete it, such as sodium dithionite $Na_2S_2O_4$ or sodium sulphite Na_2SO_3, or by using a nitrogen gas chamber. Note that the cited chemicals, while being easier to use, might react with the composition of the media. Once each well has a read-out of near to 0% oxygen, measure oxygen diffusing back into the system by either waiting for all the chemical substance to be consumed or by placing the plate in an oxygen saturated environment again. The speed at which the oxygen rises in each well will determine the oxygen mass transfer coefficient k_La.

First import the calibration data into the TReCCA Analyser, fill in the template and perform the wanted analysis including probably averaging and definitely slope calculation. It is important to run a slope calculation as the data will be needed for the oxygen consumption calibration.

To reach the calibrating platform, click on the button "Calibrate oxygen consumption" at the top right of the "Analysis options" tab. The TReCCA Analyser will ask for confirmation that the slope calculation has been done, as seen in Figure 25, and once "Continue with the calibration" is clicked a new pop-up window will appear, as seen in Figure 26.

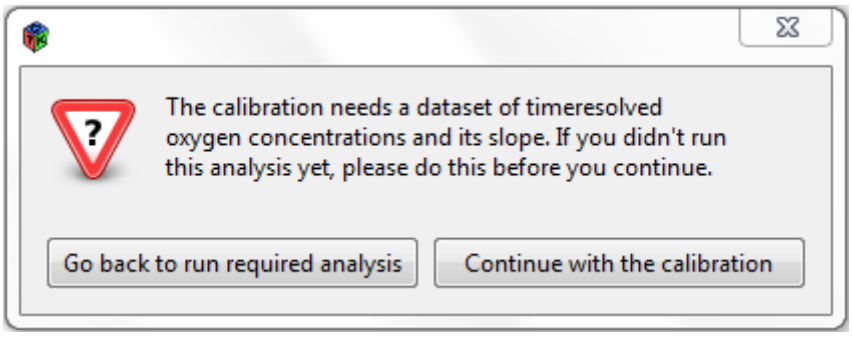


Figure 25: Pop-up before oxygen calibration

First fill in the data that should be used for the oxygen calibration. Then decide which part of the curves are linear and should be used for the calibration by filling in the corresponding time-frame and Y-axis limits of the data. Indicate the value of the oxygen in the fully saturated environment. The names that appear in this section are determined by the numbers chosen in the "Labels & colours" window under the columns "Number" of the template. Each condition that has the same number will be analysed together and plotted under the same chosen name entered in the following boxes.

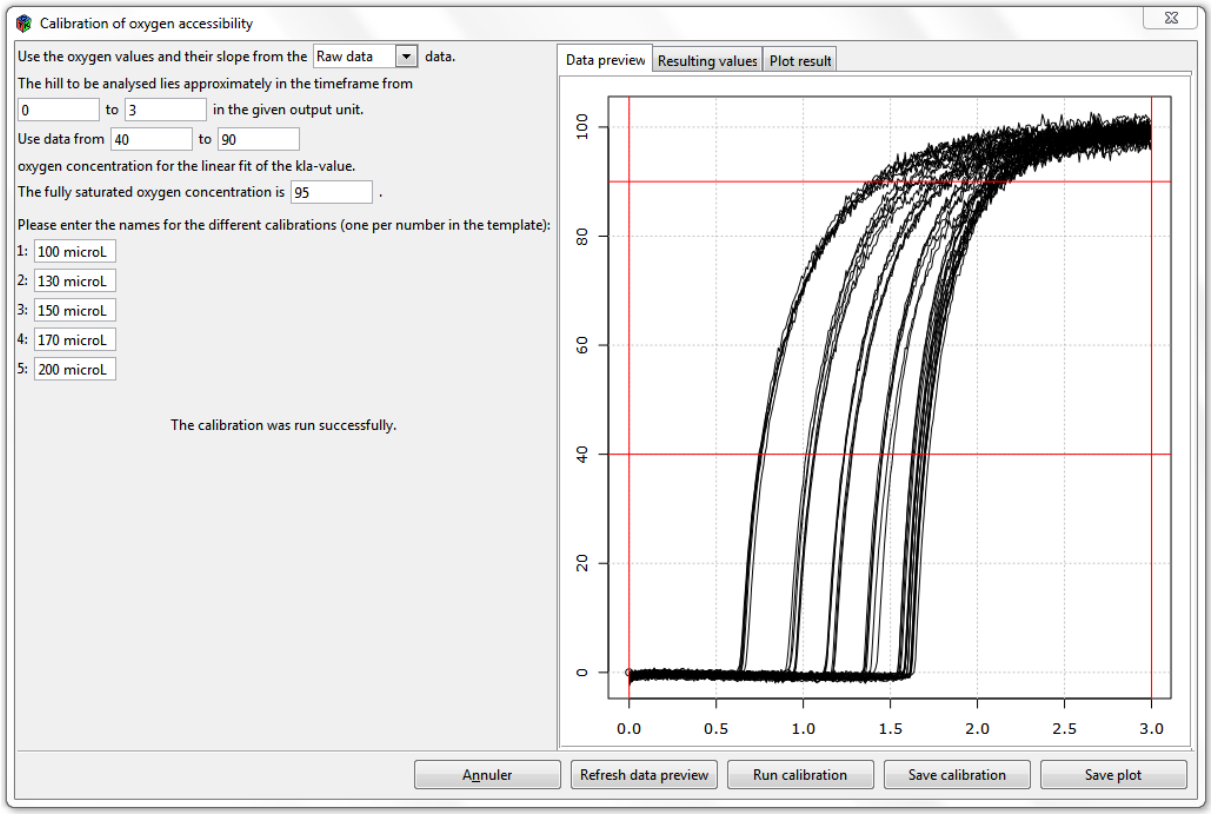


Figure 26: Oxygen calibration window - Data preview

The "Refresh data preview" button refreshes the visualisation of the selected calibration area chosen that is delimited by four red lines. Once everything is set, press the "Run

calibration" button. Two new tabs will appear next to the "Data preview": "Resulting values" and "Plot result", as seen in Figures 27 and 28 respectively. In the last tab, the diffusion flux, $d[O_2]/dt$ is depicted against the oxygen level. The line that fits this data has a slope that is equal to $-k_La$. The numerical value of the k_La for each condition, as well as the standard error and the R squared of the linear fit are presented in the "resulting values" tab.

The calibration data can be saved by pressing the "Save calibration" button. This file cannot be opened by traditional spreadsheet applications but can be loaded directly into the TReCCA Analyser as described in part **5.3.9 Oxygen consumption**. By clicking the "Save plot" button, the displayed graph will be saved in the folder currently being used for saving.

5.3.11 IC_{50} determination

The TReCCA Analyser can calculate the IC_{50} or EC_{50} of a drug automatically and in a time-resolved manner, thanks to the IC_{50} tab displayed in Figures 29 and 30.

First select the data set that should be used for the IC_{50} calculation, as seen in Figure 29. Then, choose the time points, in the input unit, between which the IC_{50} should be calculated. For the log-logistic fit of the data to be possible at every time point, it is important to select the time frame where the conditions are sufficiently different from each other. If the data cannot be fitted at one time point, then all the IC_{50} fits will not appear in the graph output. It is then necessary to change the selected time points.

In order to speed up the analysis time of the data, it is possible to calculate the IC_{50} for only some of the data, for example for every third time point. Fill in the frequency of the calculation accordingly.

Select which function to use to fit the data. For IC_{50} determination, the 4 parameter log-logistic curve is the most commonly used. An exact description of each fitting formula can be found in **6.10 IC_{50} determination**. Enter the X-axis label that should be displayed in the IC_{50} graphs.

Finally, enter the concentration of each condition used for IC_{50} determination without any unit in front of the corresponding condition. The wells that are irrelevant to the IC_{50} determination should be filled in with -1, as seen in Figure 30.

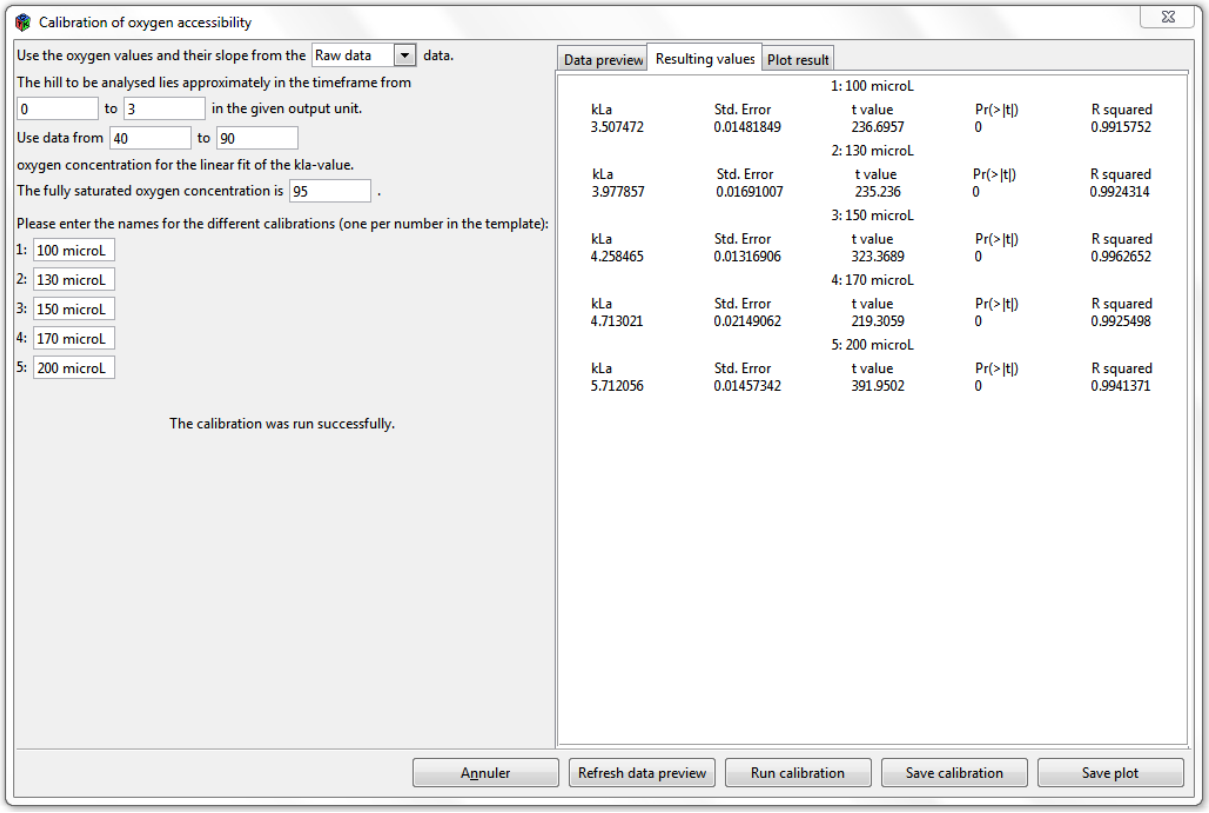


Figure 27: Oxygen calibration window - Resulting values

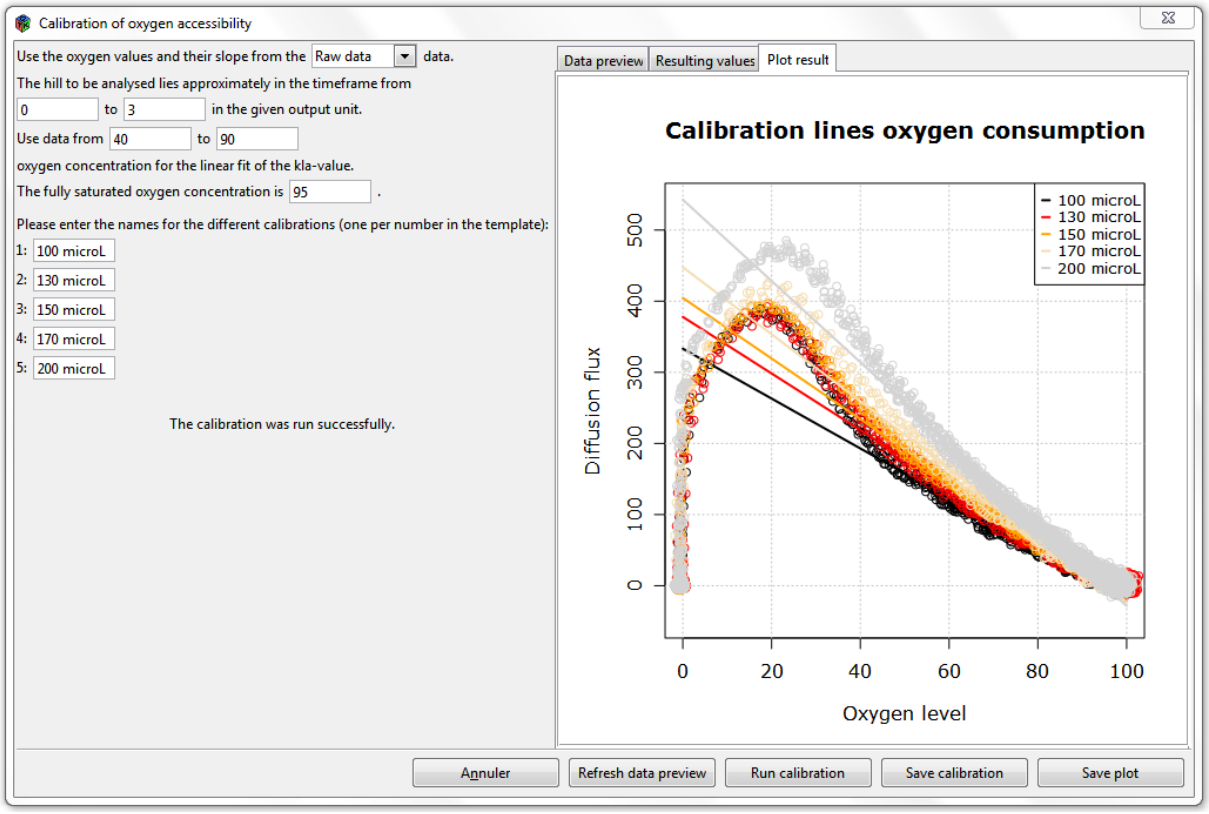


Figure 28: Oxygen calibration window - Plot result

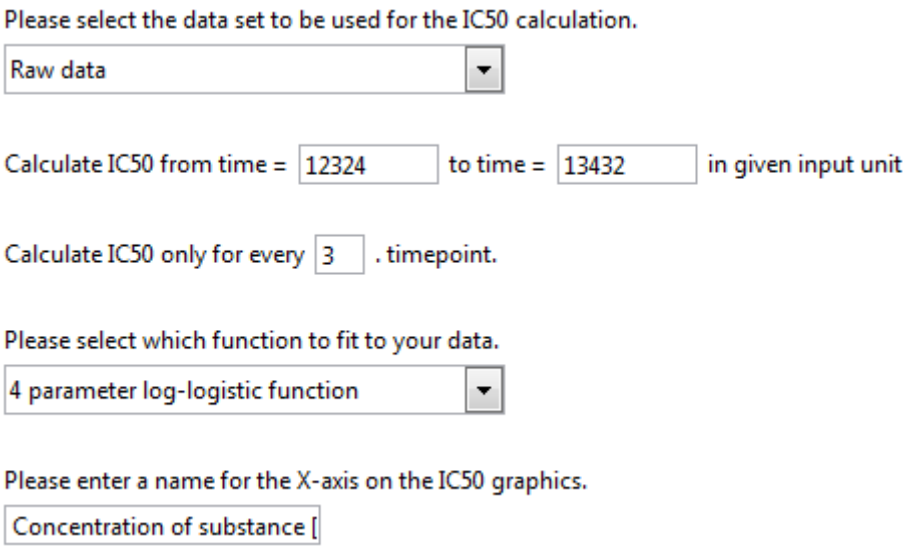


Figure 29: Analysis options - First part of the IC_{50} tab

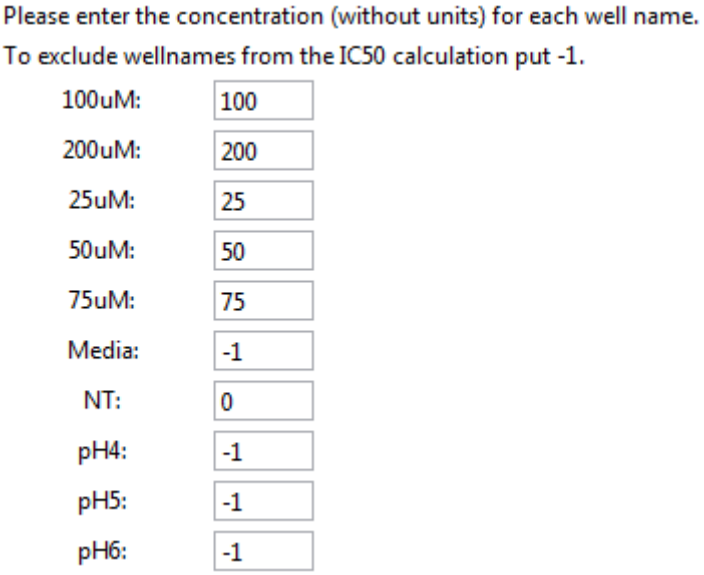


Figure 30: Analysis options - Second part of the IC_{50} tab

5.4 Graph options

In the "Graph options" tab, as seen in Figure 31, fill in the settings to determine the appearance of the graphs. All the options that are filled in this part can be changed after the graphs are visible. Many other settings can be modified after the analysis is run in the "Graph output" tab.

5.4.1 General

In the top part of the window, choose if the graphs should have titles and subtitles and if so, what these titles should be. The title will be displayed in the exact same form

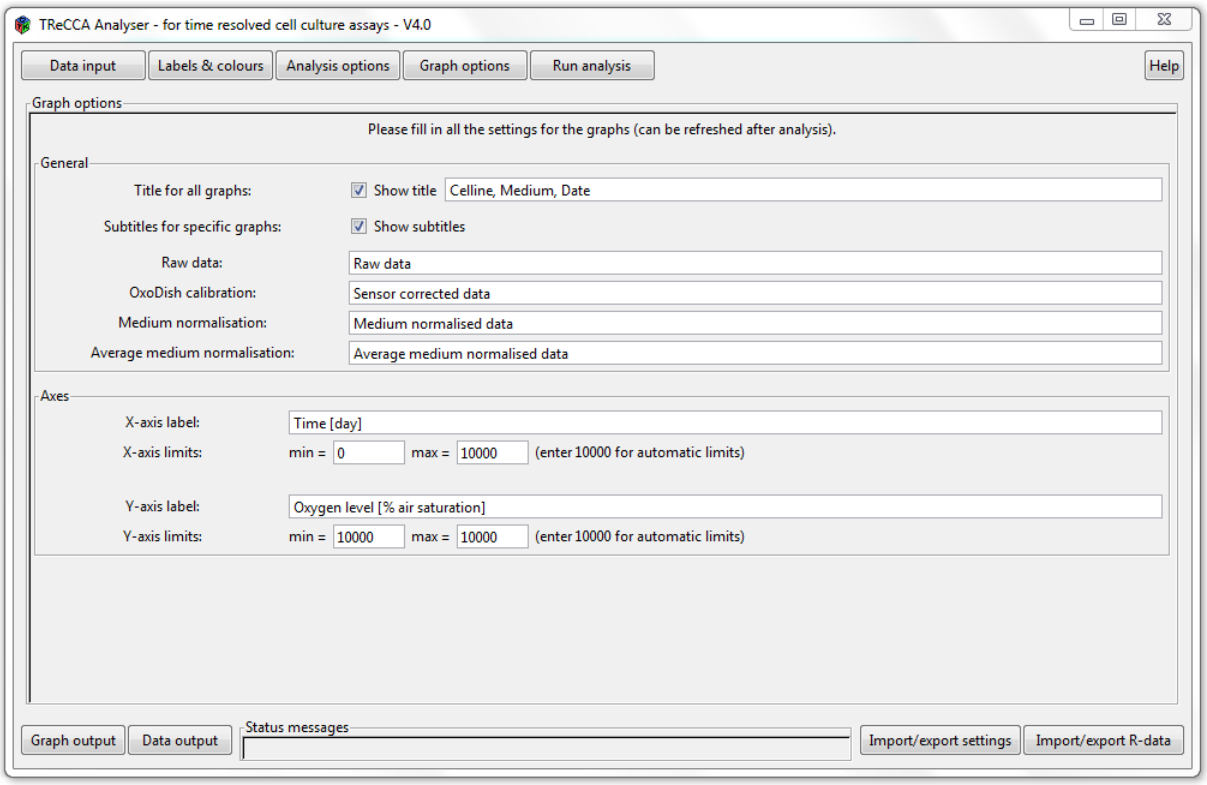


Figure 31: Graph options tab

above every single graph. The subtitles are displayed between the title and the plot on the corresponding graph.

5.4.2 Axes

Choose the label and limits of each axis. To display limits optimised by the program, choose 10 000 as minimum and maximum limit. These settings will be applied to all the graphs unless mentioned otherwise in the specific "Analysis options" tab.

5.5 Run analysis

Once all the settings are set in the "Data input", "Labels & colours", "Analysis options" and "Graph options" tabs, click on the "Run analysis" button. All the settings, imported data and the template are checked for validity.

A pop-up window will appear asking the name of the results folder to be created, as seen in Figure 32. This folder will automatically be created in the current working directory and within this folder the current settings are also saved automatically. It is possible to enter a path leading inside different folders included in the working directory as long as the upper directories are already created.

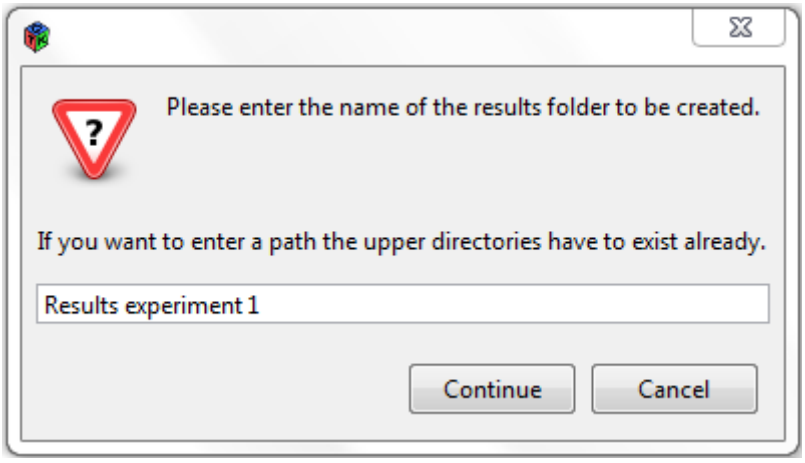


Figure 32: Pop-up window to run the analysis

If this pop-up window does not show up, then another pop-up window with a suggestion about what has to be corrected will probably appear. If this is not the case, make sure all the "Status messages" at the bottom middle of the program are clear and check the error messages in the R console. In extreme cases, restarting the program can also be an option.

After the name of the result folder is given, the analysis will start running. This will take a different amount of time depending on the size of the data sets, the variety and complexity of the analyses that have to be performed and the speed of the computer that is running. As a rough estimate, most basic analyses are usually run in under a minute and even in extreme cases, the analysis should not last more than 15 minutes.

Each time an analysis step is completed it will appear in the analysis window followed by "done", as seen in Figure 33 and the first ten rows of each data set will be printed in the R console. Under Windows the messages and data sets will appear progressively as the analysis is performed, under Mac all the information will appear at once as soon as all the analysis steps are finished. Once the program has finished running, press the "Close" button and the raw data graph in the "Graph output" tab should be visible.

5.5.1 Graph output

After running the analysis, the "Graph output" tab should be opened automatically as seen in Figure 34. In this tab it is possible to customise many more items on the graphs and export them by using the buttons and drop-down menu at the bottom of the screen. To the right, the different buttons select which calculated data sets are displayed in the graphical area. To the left, a list of checkboxes are visible which select which lines are displayed on the graphs.

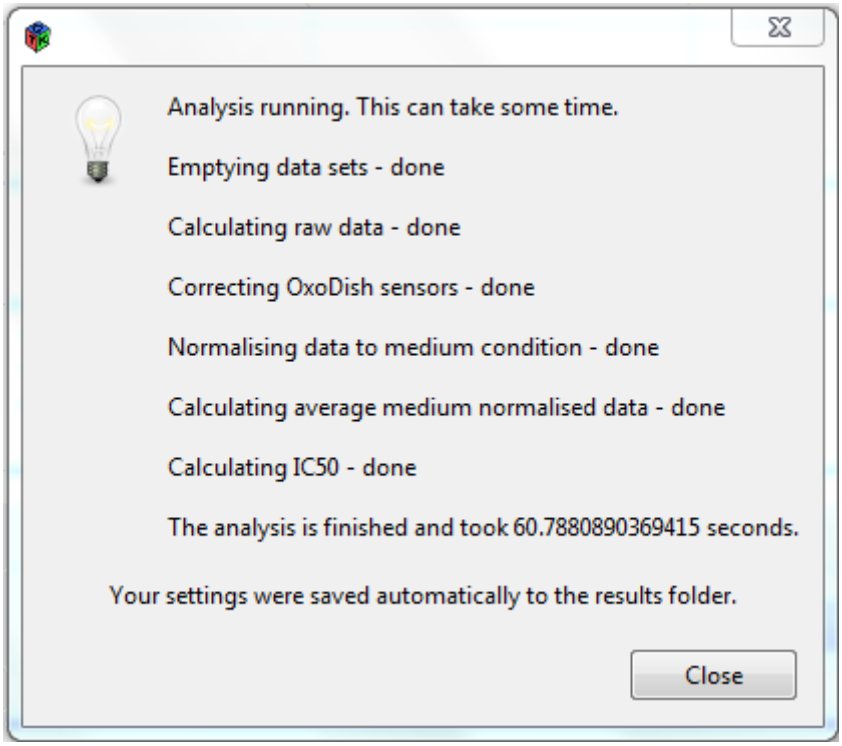


Figure 33: Window seen while the analysis is running

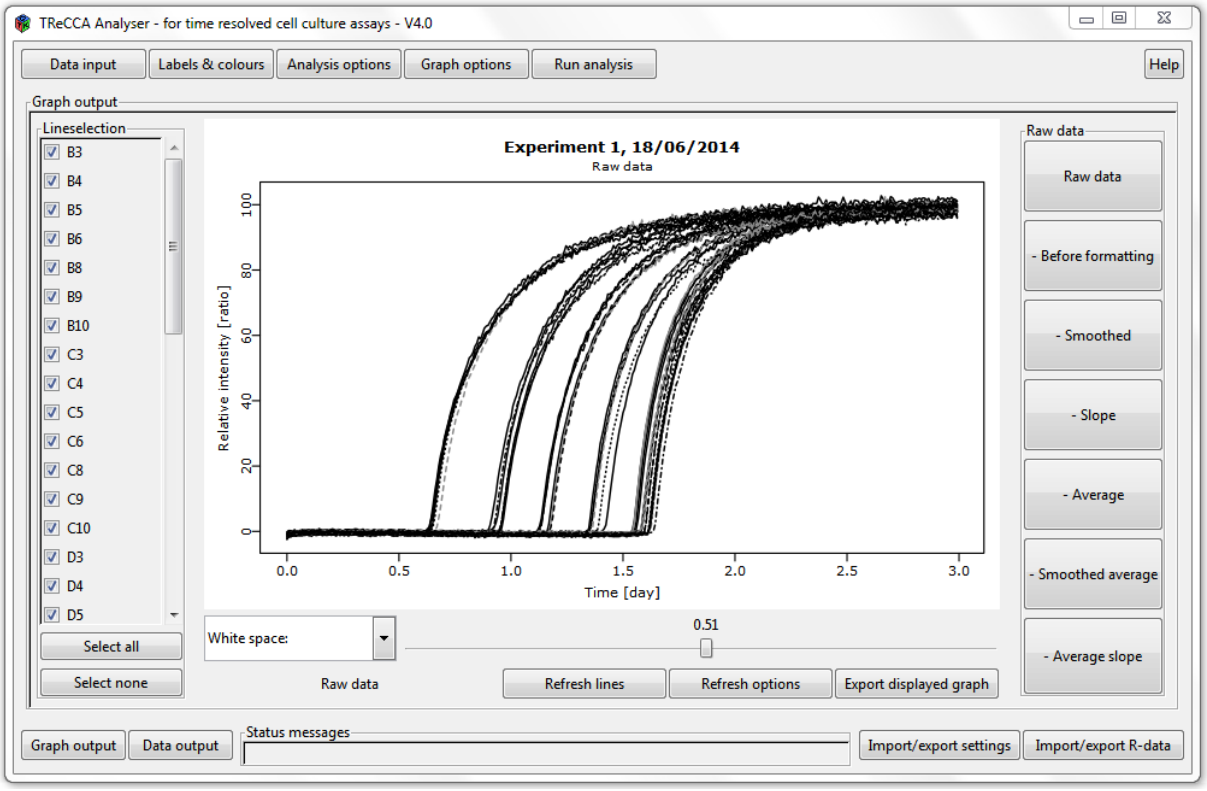


Figure 34: Graph output tab

To change options that were set previously in the "Graph options" tab, change them

in the corresponding tab and then press the "Refresh options" button for the changes to be applied. To change colours, line types or names, change the template without running the analysis again, as long as the changes do not influence the calculations (many of the analysis options use the names or numbers in the template as a basis for calculation, see the corresponding analysis details).

Graph customisation

The drop-down menu at the bottom of the screen allows the customisation of different graphical parameters. Select the parameter to be modified and then use the scroll bar or the second drop-down menu that appears to modify it. The graphs will only be modified once the slider is set, and it is also possible to click on the bar directly to skip through bigger steps instead of pulling the slider. The different possible customisations are as follows.

- **Point size:** Changes the point size of all the text on the graphs (title, subtitle, label descriptions, legend) ranging from 5.0 to 25.0.
- **Legend columns:** Number of columns used to display the legend (if appropriate) ranging from 1 to 10.
- **Legend position:** Determines the presence of a legend or not and its position. The possible options are: no legend, below the plot area, to the right of the plot area, top left, top middle, top right, middle right, bottom right, bottom middle, bottom left, middle left.
- **Line width:** Sets the width of the lines on the graph (grid lines and frame included) ranging from 0.5 to 4.0.
- **Grid colour intensity:** Picks the intensity of the colour of the grid in percentage, so ranging from 0 (no grid) to 100 (dark grey grid).
- **Error colour intensity:** For the graphs where the standard deviation is calculated (average graphs, slope calculation and time-resolved IC_{50}), changes the intensity of the error display in percentage, so ranging from 0 (no error) to 100 (black error).
- **White space:** Varies the amount of white space around the graph and how compact the representation is, ranging from 0.00 to 1.00. This option is present so that the exported graphs can be used directly for power point presentations as well as for graphs that are part of a figure in a publication.

Line selection

The line selection determines which lines are displayed on the graph. When selecting the lines to be displayed (also for the select all/none buttons) click the "Refresh lines" button to show the selected lines on the actual graph.

Graph selection

The buttons on the right of the screen determine which graph is represented in the plot area. Only graphs of the analyses that have been run can be represented. In the case of the average graphs where the standard deviation was calculated, an extra tick box appears next to the graph customisation drop-down menu. Selecting it will display the standard deviations of the averages of the curves as a grey shade behind the averaged line. For the slope calculation, the standard deviation tick box is also present and enables showing the error of the linear fit on the graphs. For the time-resolved IC_{50} data, the error of the fit can also be displayed. In the case of the display of the sigmoidal fits for the IC_{50} calculation, extra tick boxes appear above the customisation drop-down menu, allowing for a further customisation of the graphs. The first line allows to differentiate IC_{50} fittings by varying:

- **Colour:** Each fit is displayed in a different colour, the concentration points are represented as empty coloured circles.
- **Line type/symbols:** All the lines and symbols are black, the line types and symbols vary from time-point to time-point.
- **Nothing:** All the lines and symbols are black, the lines are all "solid" and the symbols are all represented as empty circles.

Exporting graphs

To export graphs, click on the "Export displayed graph" button, at the bottom right of the plot area, and give the file name and size of the desired output (size in inches). The choice of the size of the file will not influence the size of the text on the graph, so make sure that the text on the graphs still fits properly after export. To change the output file type, change the ending to either .pdf (Portable Document Format), .ps (PostScript format), .svg (Scalable Vector Graphics) or .png (Portable Network Graphics). The files will automatically be saved to the folder specified when running the analysis, included in the working directory.

5.5.2 Data output

The "Data output" tab on the bottom of the screen contains one line for every data set that was created during the analysis as seen in Figure 35. First fill in the appropriate column separator and decimal separator. Then select which data sets to export by clicking the corresponding tick boxes and give them file names ending with .csv. Click the "Export Files" button at the bottom right of the screen and the files will all be written into the specified result folder. A pop-up window will confirm the success of the export.

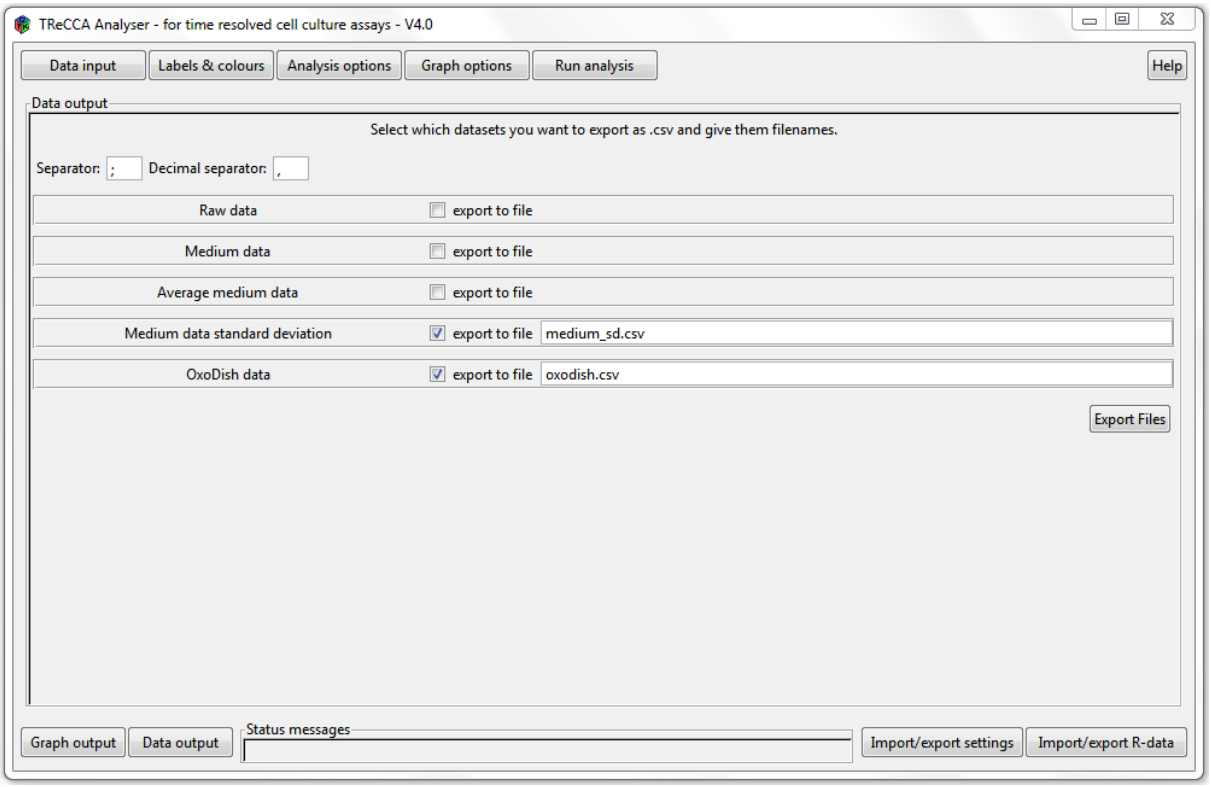


Figure 35: Data output tab

5.5.3 Import/export settings

In order to make it easier to rerun the analysis, it is possible to save all the settings chosen in the program. Click on the "Import/export settings" button at the bottom right of the screen and the pop-up window displayed in Figure 36 should appear. The file will be saved in the working directory. Type a file name ending with .txt (for example "Settings_Experiment1.txt") or a path included in the working directory followed by the file name (for example "ResultsA/Settings_Experiment1.txt"). These settings will also be saved automatically when you click the "Run analysis" button under the name "autosave_settings.txt".

The "Import/export settings" button, can also be used to load previously saved settings by clicking the "Load" button and selecting the settings file. The layout of the program will then be changed accordingly. Even after importing the saved settings it will be necessary to load the data and the template again for the analysis to run.

It is possible to customise the default settings of the TReCCA Analyser, once it is first opened. To do this, set all the settings as wished and then save them using the "Import/Export settings" button under the name "default_settings.txt", thereby replacing the existing file. The next time the TReCCA Analyser is opened, the settings should be the way they were last left.

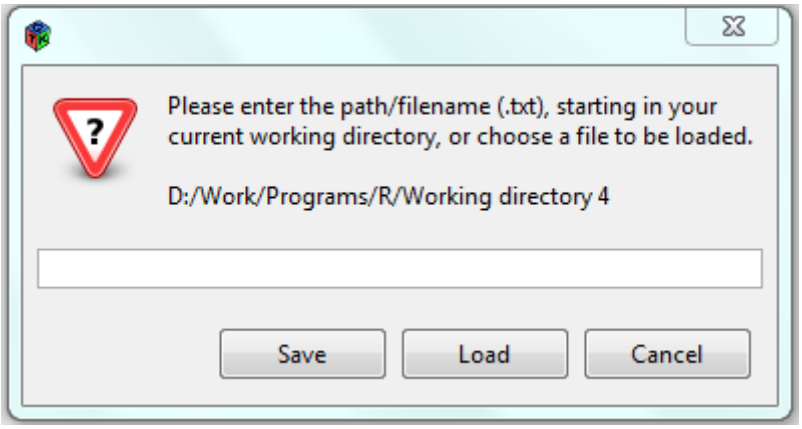


Figure 36: Pop-up for saving / loading settings.

5.5.4 Import/export R-data

To save all the data that has been imported and analysed, in order to possibly replot graphs without rerunning the analysis for example, press the "Import/export R-data" button. The corresponding file should end with ".RData" and will be saved in the working directory, as described by the pop-up window.

To load previously saved R-Data, click on the "Load" button and select the correct file. The analysed data sets will once more be available in the R-console and for plotting.

6 Technical details

In this section more details concerning the mathematical analysis of the data are presented, sorted for each analysis option available in the tab "Analysis options". For more precise information, the actual code of the program is freely available.

6.1 Average and standard deviation

The average and standard deviation are calculated according to standard statistical formulas, using the predefined functions of R. The function for the average is `mean(numerical vector)` (see Equation 6.1), where n is the number of averaged points and x_i the initial value of the replicate i. The function for the standard deviation is `sd(numerical vector)` and calculates the sample standard variation (see Equation 6.2), where $\bar{x}$ is the average for each time point.

$$mean\,(\vec{x}) = \frac{1}{n}\sum_{i=1}^{n} x_i \tag{6.1}$$

$$sd\,(\vec{x}) = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2} \tag{6.2}$$

6.2 Normalisation

The normalisation first calculates the average m_t of all normalisation wells on one plate at each time point (see Equation 6.1). Every data point from this plate is then normalised according to Equation 6.3, where M is the target value for the normalisation wells, x_t the initial value for each time point and y_t the value after normalisation for each time point.

$$y_t = \frac{x_t \cdot M}{m_t} \tag{6.3}$$

6.3 OxoDish sensor calibration

The OxoDish sensor calibration can be divided into two distinct steps. First, all the sensor read-outs inside one plate are brought to a common value, and in a second step the sensor read-outs are homogenised from plate to plate.

6.3.1 Step 1: Homogenising the sensor read-outs of each plate

The first part of the sensor correction corrects each sensor read-out from an OxoDish (so usually 24 independent read-outs) using an empty well sensor read-out from the beginning of the measurement as reference (also usually 24 reference read-outs).

The calibration curve for the conversion of the phase to the oxygen level is a complex equation (which is the property of PreSens Precision Sensing GmbH), which can be modelled by the exponential Equation 6.4 quite accurately, where P is the Plateau of the model and S the Span. The values of P and S vary from lot to lot as presented in Table 2 (see part **5.3.4 OxoDish sensor calibration**).

$$x = P + S \cdot e^{z} \tag{6.4}$$

In order to make the calibration curve linear and thereby allow a correction by multiplication and division, all the data is first converted to logarithmic values as described in Equation 6.5.

$$z = \ln\left(\frac{x - P}{S}\right) \tag{6.5}$$

Then, instead of using a discrete point as empty well reference value, all the values for each sensor included in a certain time frame are averaged and taken into account. This time frame is chosen by the user and ranges from the time points t_1 to t_n, where t_n is the last time point at the end of the sensor calibration and n the number of points averaged for calibration. The mean read-out over the time frame of each sensor s is then described by Equation 6.6.

$$m_s = \frac{1}{n}\sum_{i=0}^{n-1} z_{t_{1+i},s} \tag{6.6}$$

The mean read-out value for the total plate m_p is determined by averaging the average read-outs m_s for each sensor, as described in Equation 6.7, where w_p is the number of wells per plate.

$$m_p = \frac{1}{w_p}\sum_{j=1}^{w_p} m_j \tag{6.7}$$

The corrected values $\tilde{x}$ are then calculated according to Equation 6.8, whereby the data, which is still in a logarithmic scale, is then also reconverted back to the actual data values through the exponential function.

$$\tilde{x}_{t,s} = P + S \cdot e^{\frac{m_p}{m_s} \cdot z_{t,w}} \tag{6.8}$$

6.3.2 Step 2: Setting the average read-out to a defined target

In a second step, the corrected values $\tilde{x}$ are linearly normalised to a target value T chosen by the user according to the experimental conditions. The read outs of the correction time frame for each sensor $m_{c,s}$ are averaged according to the Equation 6.9.

$$m_{c,s} = \frac{1}{n} \sum_{i=0}^{n-1} \tilde{x}_{t_{1+i},s} \tag{6.9}$$

The resulting corrected oxygen values at the time point t and for the sensor s are then $y_{t,s}$ (Equation 6.10).

$$y_{t,s} = \frac{\tilde{x}_{t,s} \cdot T}{m_{c,s}} \tag{6.10}$$

6.4 OxoPlate oxygen conversion

Prior to the data processing by the TReCCA Analyser, the user has to divide the intensity measurement of the indicator luminophore by that of the reference measurement for each time point in a classical spreadsheet application. The resulting data (called $\mathrm{I_R}$) can then be loaded to the TReCCA Analyser to be converted to oxygen values.

The OxoPlate oxygen conversion is performed per plate using the two calibration conditions that expose the sensors to 0% and 100% oxygen in percentage of air saturation. The TReCCA Analyser first calculates the average of all 0% and 100% oxygen wells for each time point (again using Equation 6.1), thereby determining the values over time of $k0_t$ or $k100_t$ respectively. Every data point is then converted according to the Equation 6.11, where F is the unit conversion factor and x_t the value $\mathrm{I_R}$ over time.

$$y_t = F \cdot 100 \cdot \frac{\frac{k0_t}{x_t} - 1}{\frac{k0_t}{k100_t} - 1} \tag{6.11}$$

6.5 HydroPlate pH conversion

Just as in the case of the OxoPlate, prior to the data processing by the TReCCA Analyser, the user has to divide the intensity measurement of the indicator luminophore by that of the reference measurement for each time point in a classical spreadsheet application. The resulting data (called $\mathrm{I_R}$) can then be loaded to the TReCCA Analyser to be converted to pH values.

The HydroPlate pH conversion is performed per plate using 6 calibration conditions that expose the sensors to different pH conditions covering the range of pH 4.0 to pH 9.0. The TReCCA Analyser first calculates the average of the calibration pH wells for each time point (again using Equation 6.1). These values are fitted using a four parameter

logistic function curve over time (the function L4 of the drc package), as described in Equation 6.12.

$$f(x) = c + \frac{d-c}{1+\exp\left(b\left(\log\left(x\right)-\log\left(e\right)\right)\right)} \tag{6.12}$$

From this fit, the calibration constants $I_{min} = d$, $I_{max} = c$, $dpH = 1/b$, $pH_0 = log(e)$ are determined. Every data point is then converted according to the Equation 6.13, where x_t is the value I_R over time.

$$y_t = ln(\frac{I_{min,t} - I_{max,t}}{x_t - I_{max,t}} - 1) \cdot dpH_t + pH_{0,t} \tag{6.13}$$

6.6 Data smoothing

The data smoothing uses the built in mean function, but in this case an average of several points over time is calculated in order to reduce fluctuations according to the formula given in Equation 6.14, where n is the number of time points to average. Every time point is thereby replaced by the average of the time point and $(n-1)/2$ neighbours on either side. This causes the loss of $(n-1)/2$ data points in the beginning and the end of the measurement, as these points do not have the necessary neighbours.

$$y_t = \frac{1}{n} \sum_{i=t-\frac{n-1}{2}}^{t+\frac{n-1}{2}} x_t \tag{6.14}$$

6.7 Numerical slope

For the numerical slope calculation, it is usually necessary to smoothen the data first to get rid of the noise. The TReCCA Analyser always uses a smoothed data set for the calculation, but the number of points used or the smoothing can be set to 1 in which case no smoothing will actually occur. For the slope calculation, a certain number of points n on either side of the currently processed time point x are used for a linear model fit (as described by Equation 6.15). The linear model fit is a built in function of R and yields the slope m and the corresponding residual ϵ which is displayed as the standard deviation on the graphs.

$$(y_{x-n}, \cdots, y_{x+n}) = m \cdot (t_{x-n}, \cdots, t_{x+n}) + c \tag{6.15}$$

6.8 Oxygen consumption

As already described in the part **5.3.9 Oxygen consumption**, the calculations implemented in this part of the TReCCA Analyser are based on previous publications (Eyer et al., 1995; Hermann et al., 2003; John et al., 2003) and can only be used if the experimental conditions fit the prerequisites of the model described in these publications. For example, in our experimental set-ups using 96-well plates, this is the case if the solutions are mixed

over time, but not the case if they are not shaken. The first step in knowing if the model applies or not is to see whether for at least certain oxygen saturation values, the oxygen saturation value is proportional to the rate of change of the oxygen saturation value when the oxygen consumption rate is 0. This is described in more detail in parts **5.3.10 Oxygen consumption calibration** and **6.9 Oxygen consumption calibration**.

When the model applies, the oxygen uptake rate (OUR) is then described by the Equation 6.16, where $[O_2]$ is the measured oxygen concentration, $[O_2^*]$ the concentration of the saturated liquid phase and k_La is the oxygen mass transfer coefficient. For the OUR to be correct, it is essential that the k_La and the $d[O_2]/dt$ have the same time unit.

$$OUR = k_La\left([O_2^*] - [O_2]\right) - \frac{d\,[O_2]}{dt} \tag{6.16}$$

6.9 Oxygen consumption calibration

The calculations implemented in the TReCCA Analyser are based on the assumption that the general formula of the oxygen balance is as described in Equation 6.17, where $[O_2]$ is the measured oxygen concentration, $[O_2^*]$ the concentration of the saturated liquid phase and k_La is the oxygen mass transfer coefficient. The change in oxygen over time is equal to the diffusion of oxygen into the well minus the amount of oxygen consumed by the cells, yeast, bacteria or chemical reaction.

$$\frac{d\,[O_2]}{dt} = k_La\left([O_2^*] - [O_2]\right) - OUR \tag{6.17}$$

To perform the oxygen consumption calibration, it is necessary to work under conditions where the $OUR = 0$, which means by working with wells without any cells, yeast, bacteria, enzymes, etc. Equation 6.17 can then be simplified to Equation 6.18, where $k_La[O_2^*]$ is a constant.

$$\frac{d\,[O_2]}{dt} = k_La[O_2^*] - k_La\,[O_2] \tag{6.18}$$

The oxygen concentration $[O_2]$ is set to 0 by using either nitrogen or chemicals that react with oxygen, as described in part **5.3.10 Oxygen consumption calibration**, and then the diffusion of oxygen back into the solutions is measured over time until it reaches the level of the ambient saturation.

The rate of oxygen change over time $d[O_2]/dt$ can be calculated using the slope function of the TReCCA Analyser. Plotting this slope against the actual oxygen value $[O_2]$ and fitting a linear model then yields k_La and the corresponding residual ϵ for the fit. Using the plots that are displayed in the oxygen consumption calibration interface (Figure 26, 27

and 28) it is possible to determine if the model for the oxygen balance in the experimental set-up is valid and if so, to set the range for the linear fit in order to exclude the initial or final phases of the measurement.

6.10 IC_{50} determination

For the IC_{50} calculation the TReCCA Analyser uses concentrations assigned to the labels of the data by the user. It is also possible to select how many time points to skip in order to reduce the run time of the calculation. From this a data set is created, where the concentrations replace the well names, fewer time points are included, and those wells which do not belong to the IC_{50} calculation are excluded. The IC_{50} is calculated for each line of the data set, which represents one time point. The possible models used for determining the IC_{50} are included in the drc package and are the two-, three-, four- and five-parameter log-logistic models as depicted in Equations 6.19, 6.20, 6.21, 6.22 respectively. The error is reduced by a non-linear least-squares method.

$$f(x) = \frac{1}{1 + \exp\left(b\left(\log\left(x\right) - \log\left(e\right)\right)\right)} \tag{6.19}$$

$$f(x) = \frac{d}{1 + \exp\left(b\left(\log\left(x\right) - \log\left(e\right)\right)\right)} \tag{6.20}$$

$$f(x) = c + \frac{d - c}{1 + \exp\left(b\left(\log\left(x\right) - \log\left(e\right)\right)\right)} \tag{6.21}$$

$$f(x) = c + \frac{d - c}{\left(1 + \exp\left(b\left(\log\left(x\right) - \log\left(e\right)\right)\right)\right)^{f}} \tag{6.22}$$

The curve of the IC_{50} fit for each time point, as well as a curve of the IC_{50} value over time are shown.

7 Trouble shooting

This section is designed to help find a fast solution to any error message or problem that may be encountered while using the TReCCA Analyser.

7.1 Error messages

Error messages regarding the settings are shown in the status messages bar in the bottom of the program window. This bar is a scrollable list. In Table 3 all possible error messages are listed in alphabetical order. A short explanation and solution to each message is presented and each error message is linked to the section in the manual it is related to for more details. Errors that prevent an action from being taken will appear as a pop-up window. The Tables 4, 5, 6 and 7 give suggestions to solve each problem.

7.2 R console

Some error messages and warnings can appear in the R console and be ignored. If after solving all the error messages in the scrollable list status messages bar and all the pop-up errors, something still is not functioning correctly, double check whether the data is cut properly, whether there is non numerical data left in the data set (this can be checked easily using the program), whether the template contains exotic characters that might affect plotting and whether all the paths and folders given are valid. If the problem persists, it is possible to check the R console for additional complications. Many problems can then be resolved by following the instructions in the R console, by searching the error message on-line or by asking the questions on an online forum.

Error message	Chapter	Solution
Entered path doesn't lead to an existing file!	Labels & colours	Use the file chooser properly to select the template. If this error occurs when starting the program, the default template from the Sample Project folder is missing. This is fine but the error will always occur upon start up.
Entered paths don't lead to existing files!	Data input	Use the button showing a folder to open a file chooser dialogue and select the data (typing the file name/path can lead to typing errors), avoiding exotic characters in the pathways. When importing Settings, make sure the input data is still in the same place on the computer.
Given last value for sensor calibration can't be found in imported data!	OxoDish sensor calibration	Make sure the time point is included in the data file. A rough time point is not enough - the exact time point has to be given in the input unit.
Given name for normalisation is not in the labels list!	Normalisation	The name for the normalisation condition has to be in the "Labels & colours" template in exactly the same spelling and capital/small letter combination.
Given name for 0 % oxygen wells is not in the labels list!	OxoPlate oxygen conversion	The name has to be in the "Labels & colours" template in exactly the same spelling and capital/small letter combination.
Given name for 100 % oxygen wells is not in the labels list!	OxoPlate oxygen conversion	The name has to be in the "Labels & colours" template in exactly the same spelling and capital/small letter combination.
Max. number of plates is 10!	Data input	The number of plates has to be a whole number between 1 and 10 (included).
Please enter a number for end of IC_{50} calculation!	IC_{50} determination	Make sure the exact time point for the end of the calculation interval given in the input unit is included in the data file.
Please enter a number for normalisation target!	Normalisation	Enter a target value for normalisation without any measurement unit and . as a decimal separator.
Please enter a number for Plateau!	OxoDish sensor calibration	Enter a number with . as decimal separator.

Please enter a number for sensor calibration target!	OxoDish sensor calibration	Enter a number with . as decimal separator.
Please enter a number for Span!	OxoDish sensor calibration	Enter a number with . as decimal separator.
Please enter a number for start of IC_{50} calculation!	IC_{50} determination	Make sure the exact time point for the start of the calculation interval given in the input unit is included in the data file.
Please enter a number for the error of the k_La value!	Oxygen consumption	Enter a number with . as decimal separator.
Please enter a number for the k_La value!	Oxygen consumption	Enter a number with . as decimal separator.
Please enter a number for the saturated oxygen concentration!	Oxygen consumption	Enter a number with . as decimal separator.
Please enter a number for the slope fitting points!	Numerical slope	Enter a whole number superior or equal 1.
Please enter a number for the unit conversion factor used for the oxygen consumption!	Oxygen consumption	Enter a number with . as decimal separator. 1 for no conversion.
Please enter a number for the unit conversion factor!	OxoPlate oxygen conversion	Enter a number with . as decimal separator for the unit conversion of the luminescence data to the target unit.
Please enter an odd number for the curve smoothing!	Data smoothing	Enter a whole odd number superior or equal to 1.
Please enter a number > 1 for sensor calibration range!	OxoDish sensor calibration	Enter a number of time points for sensor correction superior to 1 (the last time point is included and one point is insufficient for the correction).
Please enter a positive number for Header length!	Data input	If you do not have a header in your file(s) select "No" so the number will be ignored. Otherwise enter a positive whole number.

Please enter a positive number for last calibration value!	OxoDish sensor calibration	The time point specified for the last calibration point must be a positive number found in the time column of the data set (in the input unit).
Please enter a positive number for number of plates!	Data input	The number of plates has to be a whole number between 1 and 10 (included).
Please enter a positive number for skipping points in IC_{50} calculation!	IC_{50} determination	Enter a whole positive number for the frequency of IC_{50} calculation. For all time points to be taken into account choose 1.
Please enter numbers for X-axis limits!	Graph options	Enter any number with . as decimal separator and 10 000 if for automatic axis limits.
Please enter numbers for Y-axis limits!	Graph options	Enter any number with . as decimal separator and 10 000 if for automatic axis limits.
Please enter numerical values $>$ 0 or -1 only for the IC_{50} doses!	IC_{50} determination	Enter the concentrations as positive numbers with . as a decimal separator or -1 to exclude a condition from the IC_{50} calculation.
Please enter numerical values $>$ 0 or -1 only for the pH values!	HydroPlate pH conversion	Enter the pH values as positive numbers with . as a decimal separator or -1 to exclude a condition from the pH conversion.
Please enter positive numbers for wells!	Data input	There has to be a positive whole number in every field next to the rows for each imported plate. For several plates, just one number in the first field is not enough!
Please enter positive numbers or 0 for spare columns!	Data input	Enter a positive whole number for the data formatting or 0 to not delete any columns.
Please enter positive numbers or 0 for spare rows!	Data input	Enter a positive whole number for the data formatting or 0 to not delete any rows.
Please enter proper filenames for all the data sets to be exported!	Data output	Enter file names for all the chosen files to be exported. They should all end with ".csv".
Please only enter numerical values for the formatting parameters!	Basic data formatting	Enter numbers with . as decimal separator in the output unit for each formatting parameter.

Starting time point of IC_{50} calculation has to be before end point!	IC_{50} determination	The IC_{50} calculation interval time points (in input unit) are not in the right order.
The given time point for end of IC_{50} calculation can't be found in imported data!	IC_{50} determination	Make sure the exact time point for the end of the calculation interval given in the input unit is included in the data file.
The given time point for start of IC_{50} calculation can't be found in imported data!	IC_{50} determination	Make sure the exact time point for the start of the calculation interval given in the input unit is included in the data file.
There are not as many time points for calibration as you want to include!	OxoDish sensor calibration	There are not enough time points prior to the starting point specified. Enter a smaller number of time points or set a higher starting time point.
You are about to remove the stop time point of the sensor correction!	Basic data formatting	By proceeding with this basic data formatting step, the time-point used for OxoDish sensor calibration will be removed.
Your imported data doesn't contain enough time points for smoothing!	Data smoothing	Reduce the number of points set for smoothing or prepare a data set with more time points.
Your imported data doesn't contain enough time points for the slope calculation!	Numerical slope	Reduce the number of points set for the slope calculation or prepare a data set with more time points.

Table 3: Error messages shown in the status messages bar in alphabetical order

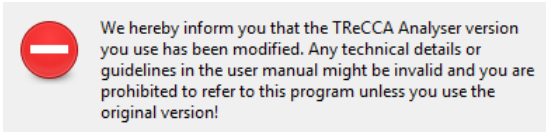
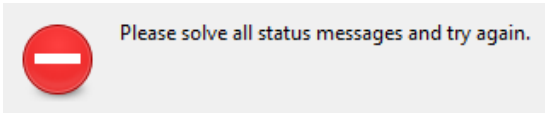
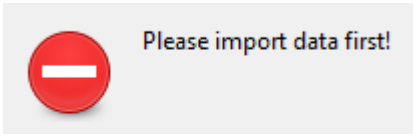
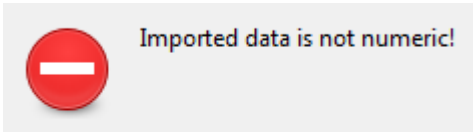
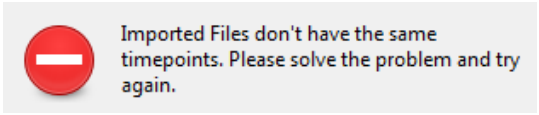
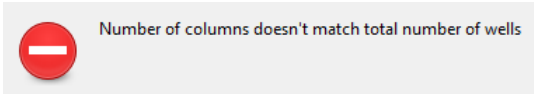
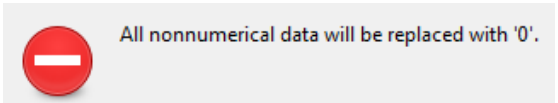
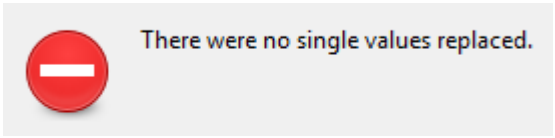
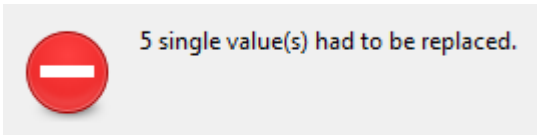
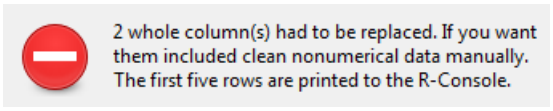
General errors	
	The script for the TReCCA Analyser that you are using has been modified. Download the original files again to work with the correct version.
	Error upon data import or when trying to run the analysis. Some of the settings are invalid and there are errors in the status messages bar. Please look up solutions in Table 3.

Table 4: Pop-up errors and solutions: General errors

Data input	
	Error upon trying to automatically fill the template's well number column or trying to run the analysis. Either the data is not imported yet or a severe error message occurred upon trying to import it. The data input is also cleared whenever settings are loaded. Please go to the data input interface and press import data and if necessary solve the other error messages.
	Error upon trying to import data without getting rid of non numerical data. Your data contains non numerical data. Either change your tick for the data cleaning or clean the data manually.
	Error upon trying to import data from several plates. This means that the time columns in your files are not identical. This can be due to different line numbers in the files. Otherwise it is recommended to analyse the plates independently.

	Error upon trying to import data. The sum of the well numbers you specified + 1 for the time column doesn't match with the total number of columns in the (merged) data you tried to import. Please check the columns again and change the numbers accordingly.
	Warning pop-up that all the data that is not recognised by the program as a number will be replaced by 0. This will not affect your original data files.
	Information pop-up. The files imported contained data that was all recognised as numerical, no data point was replaced by 0.
	5 discrete value(s) are not recognised as numerical. Accept that they will have the value 0 or check out the original files and modify them. The files could contain text such as "Error", "> 9.0 pH" or numbers with the wrong decimal separator.
	2 whole column(s) are not recognised as numerical. Accept that they will have the value 0 or check out the original files and modify them. The columns could contain values recognised as text such as "Error", "> 9.0 pH" or numbers with the wrong decimal separator.

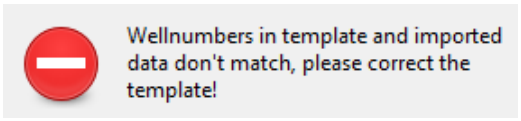
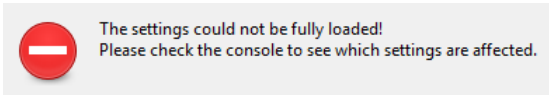
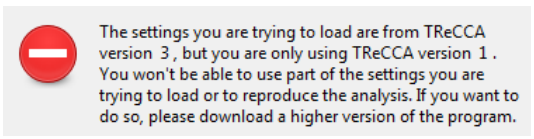
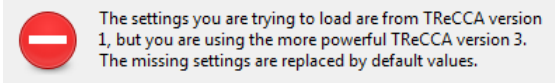
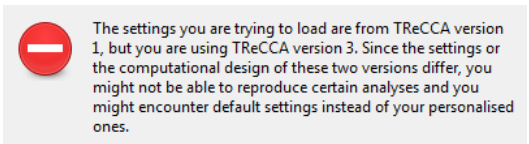
	Error upon trying to import data / load template. The well numbers in your data and in the currently loaded template do not match. If you haven't filled in the "Labels and columns" tab yet this error can be ignored. Otherwise you should check all the files again, or import the data and use the auto fill button for well names in the template.

Table 5: Pop-up errors and solutions: Data input

Settings and R-Data import	
	Error upon trying to import settings. Either the file is corrupt or you are using settings from an earlier version of the TReCCA Analyser. Check the R console to see which settings are affected or go through the different settings manually in the program.
	Error upon importing settings. Some settings available in version 3 of the TReCCA Analyser were not available in the version 1 or 2. Download the most recent version of the TReCCA Analyser for the optimal functionality, or accept the missing settings and check through the settings manually.
	Error upon importing settings. Accept the default settings as they are or go through the settings manually to check them all.
	Error upon importing settings. The computational design has changed from one version to the next and therefore some analyses might not be available. Go through the settings manually to check them all.

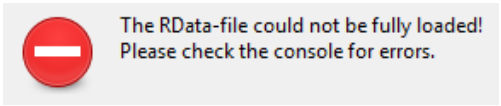
	Error upon importing an RData file. Make sure that the file is saved as .RData or check the console for further possible errors.

Table 6: Pop-up errors and solutions: Settings and R-Data import

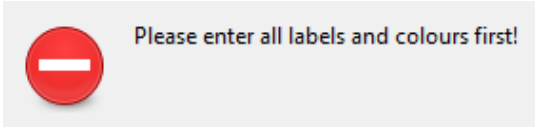
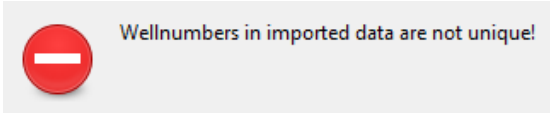
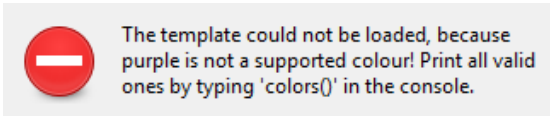
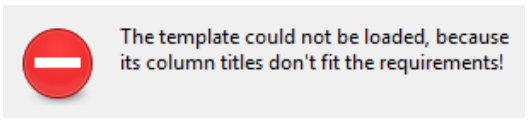
Labels and Colours errors	
	Error upon trying to run the analysis. A proper "Labels & colours" template is not loaded yet, or has been cleared by loading settings. Please go to the "Labels & colours" tab and click the load template button to open a file chooser dialogue or create a new template using the autofill buttons.
	Error upon trying to import data. The names of the columns are not unique. This can happen easily by merging the data manually. Please change the names accordingly or use the automated file merging to prevent this error.
	There are colours in the template which are not recognised by the program. Please only use colour names (and not numbers), check there are no white spaces after the colour name or spelling mistakes. All the valid colours can be found by typing "colours()" in the R console.
	Error upon trying to load a template. This means that the first row in the template does not contain the right names. It might help to remove all the quotation marks from the csv-file. Another reason might be the separator chosen for loading the template.

Table 7: Pop-up errors and solutions: Labels and Colours

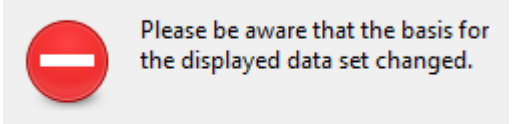
Graph output	
	Error upon visualising data after using the "rerun" button for the data analysis. Data sets used to calculate the data shown on this graph have been modified a posteriori.

Table 8: Pop-up errors and solutions: Graph output

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